EVOLVED PLATFORMS FOR VIRAL OUTGROWTH AND MOLECULAR CHARACTERIZATION OF HIV WITHIN TISSUE RESERVOIRS

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EVOLVED PLATFORMS FOR VIRAL OUTGROWTH AND MOLECULAR CHARACTERIZATION OF HIV WITHIN TISSUE RESERVOIRS

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HIV persists during long-term, effective antiretroviral therapy within reservoir cells. While CD4-positive lymphocytes are accepted as bona fide reservoirs, the contribution of other HIV-susceptible cell types to viral persistence remains unknown. ART therapy is a lifelong commitment for those with HIV, as treatment interruption induces swift rebound of peripheral viremia and progression toward AIDS. This demonstrates that persistent HIV reservoir cells are not eliminated by the virus or cleared by the immune system. However, while the memory T-cell HIV reservoir was discovered twenty years ago, strategies to specifically target these cells for destruction remain elusive. Furthermore, we know far less about HIV-susceptible cells within tissues than those in the blood, including subsets of myeloid cells such as tissue macrophages. Indeed, challenges in the study and isolation of tissue macrophages and the sheer number of organ-specific myeloid subsets has complicated unified efforts to learn whether these cells harbor HIV during treatment.

Here I present a series of experimental platforms for the study of HIV tissue reservoirs, with a focus on pulmonary alveolar macrophages of the human lung. To learn whether these cells are HIV-infected in vivo, I describe a flow-cytometry assay using fluorescence RNA in situ hybridization (FISH) that reveals individual HIV-infected primary cells in a cell suspension. These assays are amenable to multiplex

HIV antigen staining and sorting of HIV-expressing cells for RNAseq analyses. Using this FISH:FLOW tool I demonstrate a universal caveat in the industry standard cell line 8E5, used routinely as a calibration standard for clinical measurements of the HIV reservoir. Secondly, I engineered several reporter cell platforms for the outgrowth of HIV from tissue reservoirs, and use these tools to develop novel paradigms in the study of HIV replication and cellular transmission in myeloid cells. Finally, I deploy some of these cell lines in a combined HIV outgrowth pipeline to capture and characterize Clade C HIV in the blood and lungs of adults in Blantyre, Malawi. Together, these contributions will accelerate our progress toward therapies that specifically target HIV reservoir cells in the tissues, the key obstacle to an HIV cure.

BIOGRAPHICAL SKETCH

David William J. Gludish was born in Toronto, Ontario and attended Mentor College through high school. He was always a student of the sciences, and pursued an undergraduate degree in Honours Biochemistry and Biomedical Sciences at McMaster University. Upon completion of his B.Sc. in 2004, David was enthusiastic to be accepted to the graduate program in the same department at McMaster.

At the time, David probably didn't appreciate how woefully inadequate are the textbook figures at representing the true complexity of biology, an oversight he still grapples with today. He pursued the mechanisms and stem cell properties of breast tumor initiating cells, and was introduced to the power of single cell functional analyses long before such studies came of age. David defended his Masters thesis on functional genomics of breast tumor initiating cells in 2007, and set his sights on Harvard Medical School to continue pursuing the things he loved.

His research training continued at Children's Hospital Boston and Harvard Medical School, where David was privileged to work on stem cells of the adult lung, developing additional skills in the study of developmental biology and embryogenesis. His interests in animal models of human disease continued to swell, and in 2009 was excited to depart for Ithaca, NY and Cornell University.

David sought a profession at the forefront of biological innovation, and realized that training in veterinary medicine would bolster his credentials to drive valuable research in disease models. He was thrilled to be accepted to the Cornell University combined degree DVM/Ph.D. program, continuing his quest to find the questions that return simple answers like those in the textbooks.

Now with the submission of this Ph.D. dissertation, his attention turns toward training in clinical medicine and what lies beyond. So ends David's epic journey to earn a doctorate degree in the sciences.

This work is dedicated to my family. Without you, none of this work has any real meaning. And without your support, none of it was possible.

To my wife, you alone in our family knows what this takes and what it means for us. I started this journey for myself, but I finished it for you and because of you, for our baby girls, and for our future together.

> *Until one is committed, there is hesitancy, the chance to draw back-- Concerning all acts of initiative (and creation), there is one elementary truth that ignorance of which kills countless ideas and splendid plans: that the moment one definitely commits oneself, then Providence moves too. All sorts of things occur to help one that would never otherwise have occurred. A whole stream of events issues from the decision, raising in one's favor all manner of unforeseen incidents and meetings and material assistance, which no man could have dreamed would have come his way.* ***Whatever you can do, or dream you can do, begin it. Boldness has genius, power, and magic in it.***
>
> ~W.H. Murray after Goethe

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I am thrilled to have worked with Linda in the lab, though her and I do nothing but complain. Linda, you know how it's done. Thanks for keeping the lab and my life in it as productive as it could be. It's refreshing to work with someone who is great at their job.

This project and my connection to it would not be possible without the unwavering support of Dr. Henry Mwandumba and his team in Malawi, including Kondwani and Dumi. And to all those in Blantyre I haven't met, but who work with these patients and our project everyday, I am humbled and honored to collaborate with you.

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Dr. Janice Clements, many thanks for your time and support in navigating the nuances of the field. Your work and time spent with me sets a great example for how to mentor. To Dr. David Margolis, your kind words also made me feel welcome and unintimidated. To Drs. Joe Mankowski, Kelly Pate and Sarah Beck, what a breath of fresh air to meet and talk with you. I have a feeling it won't be the last time we carry on about macrophages.

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I need to thank the patients and volunteers who I have never met, and know only by their numbers written on a tube in my freezer. I am inspired and quietly humbled by the connection I feel to your cause. Without your help, HIV will never be beaten.

And finally, to my family. I offer you all that I have in repayment of the time, the care, and the sacrifices you have made to allow me to pursue this. To my mom, who knew all along she would see this through, I hope this brings you everything you sought. My thanks to my wonderful in-laws, who treat me like their own and make all the effort. To my beautiful daughters who don't know me well enough, I offer you my thanks for being patient with Dadi and always smiling. And to my wife, who has given up too much to offer me the chance to do something as great as this is, I can only give my most profound love and gratitude. We both know I could never have done this without you. This is all for you.

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LIST OF ABBREVIATIONS

27ZP	clade C viral swarm 10.27Z-PBMC
2X SSC	2X saline sodium citrate
AIDS	acquired immune deficiency syndrome
AMs	alveolar macrophages
ART	antiretroviral therapy
ATP	adenosine triphosphate
BAL	bronchoalveolar lavage
CCR5	C-C chemokine receptor type 5
CD	cluster of differentiation
CFAR	center for AIDS research
CSF	cerebrospinal fluid
CXCR4	C-X-C chemokine receptor type 4
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
env	envelope
FACS	fluorescence activated cell sorting
FISH	fluorescent in situ hybridization
FLOW	Flow cytometry
FSC	forward scatter
Gfp	green fluorescent protein
GGR	gaussia luciferase-GFP-reporter
GLuc	gaussia luciferase
GM-CSF	granulocyte-macrophage colony-stimulating factor
HIV	human immunodeficiency virus
HMDMS	human monocyte-derived macrophages
hrGFP	humanized recombinant green fluorescent protein
HuFLM	human fetal liver-derived macrophages
IL4	interleukin-4
IRES	internal ribosomal entry site
LAV	lymphadenopathy-associated virus
LTR	long terminal repeat
MdFI	median fluorescence intensity
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
NF-kB	nuclear factor kappa-B
PBMC	peripheral blood mononuclear cell
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
QVOA	quantitative viral outgrowth assay
RNA	ribonucleic acid
RNAse	ribonuclease

RRE	rev-response element
RT	reverse transcriptase
SAMHD1	SAM domain and HD domain-containing protein 1
SIV	simian immunodeficiency virus
SSC	side scatter
ssDNA	single-stranded deoxyribonucleic acid
T/F	transmitted/founder
TCID50	50% tissue culture infective dose
TIM	transmembrane immunoglobulin and mucin domain
tRNA	transfer RNA
TZM	Tranzyme
VCC	virus-containing compartments
VSV/G	vesicular stomatitis virus G glycoprotein

pref·ace

ˈprefəs/

noun

1. an introduction to a book, typically stating its subject, scope, or aims.

How do you preface a dissertation?

This dissertation is a story about one young scientist's quest for the truth about the human immunodeficiency virus. The experiments I describe below were centered on the hypothesis that, contrary to an overwhelming body of evidence, HIV persists during antiretroviral therapy in a diversity of cell types in addition to CD-4 positive lymphocytes.

The aim of this document is to summarize several years of work. But like so many projects in scientific research, this dissertation will pose more future questions than those of the present it definitively answers.

The scope of the work below spans reporter cells and macrophage biology, and the technical challenges encountered while studying HIV in these cells. My work has revealed (to me) how little we know about a powerful category of immune cells, often summarily affronted by the term "myeloid reservoirs" by members of the field. I describe what seemed to me a logical and first principles approach to the study of HIV replication in several cell types, and found myself reading literature published around the earliest days of HIV discovery, when minds

in the field were not overburdened with details and conclusions were less censored during peer review.

Long before I had sufficient data to present, I attended a Keystone HIV persistence meeting in Boston to eavesdrop. I found a group of ~160 scientists, mostly clinicians, engaging in healthy debate about substantial issues in the field. Like most clinicians, these people were focused on numbers, survival curves, and patient outcomes. As a graduate student in basic research, I admit I was unprepared for how it feels to stand somewhere between the bench and the patients, debating the outcomes that turn into standards of care. Many in the HIV cure field – and I do mean many – are the same physicians who first saw the young men emerging from the woodwork in California and New York city beginning in April 1980. They describe the sinking feeling of personally meeting the exponentially-increasing number of patients with classic AIDS-relating wasting symptoms, without knowledge of the agent or any form of treatment. It wasn't until 1987 – seven years – that the first HIV treatment, AZT, was approved. By that time, there were 50,000 reported AIDS diagnoses in the United States and hundreds of thousands more infected. The researchers in session at that Boston meeting diagnosed and 'treated' many of these first patients, but they had all trained in an era where communicable disease was largely controlled by antibiotics, and periodic influenza outbreaks were something to be feared but its symptoms were predictable and its etiology known. For the first time in a generation, modern clinical medicine faced an unknown enemy with absolutely no arsenal. Hearing these individuals speak to one another

was inspiring. While HIV has always been a field packed with controversy, these investigators had all faced the epidemic together and there was a reverence to their debate that still bore the scars of not knowing any answers when asked by a dying patient.

In a way, these stories concern us all, because this is how the clinics will look when our antibiotics no longer work. And while the HIV pandemic in some areas of the globe is "under control", the virus is driving the emergence of multidrug-resistant tuberculosis in places like sub-Saharan Africa and Asia, for reasons that are socioeconomic, biological and difficult to study. On days when you forget why you're in research or feel like tossing this or that experiment, it helps to remember that what you are doing actually matters. Working on any project in the basic sciences, we need to remember that somewhere, somebody's child is sick or dying because as a field – as a team – we have not yet reached them. Entering the field of HIV persistence, hearing the co-morbidities, the millions infected, the reasons patients can't or won't stay on their medications, the unbelievable efforts made to exhaust every avenue, you are humbled, driven, and quietly optimistic that you too can contribute.

A PhD dissertation is a bit like a teenage romance, isn't it? It consumes your thoughts, you can't sleep, you often stop eating. During your PhD you feel at once despondent, excited, furious, and sublime. It's an unreasonable and time-costly commitment toward something much greater than yourself, made in the prime of

your life. You enter as a juvenile and leave as an adult, but don't have much opportunity to look back and see whether in the process you've grown. And finally, we make decisions so much differently at the outset compared to the end. But if over the course of my studies I didn't stumble on a cure for HIV, or even finally put to rest the "simple" question of HIV persistence in alveolar macrophages, I can look back and say with confidence that I had a hand in something that may change how we think. With this dissertation, I may actually help *reach* those people.

All over the world, there are scientists who give up much of what is available to them in life to find the answers and define the truths that will one day bring relief to the sick. Despite my toughest moments in science, to have played even a small role in this effort is the opportunity of a lifetime, and for that I will always be grateful.

Structure of this dissertation

one The first chapter is an introduction to the biology of HIV, the prototype lentivirus.

two The second chapter details work to identify HIV infection at the level of single cells.

> The section in chapter 2 on Quantitative HIV biology with FISH:FLOW population analyses is published as a corresponding author paper:
>
> Wilburn KM, Mwandumba HC, Jambo KC, Boliar, Solouki, Russell DG, **Gludish DW**. *Heterogeneous loss of HIV transcription and proviral DNA from 8E5/LAV lymphoblastic leukemia cells revealed by RNA FISH:FLOW analyses.* Retrovirology. 2016 Aug 11;13(1):55

three The third chapter describes the development of multiple reporter cell lines and associated assays to rigorously study cellular transmission of different strains of HIV.

> The data in chapter 3 form the foundations of two manuscripts: (**1**) on the construction and application of TZM-gfp cells and (**2**) on the development of novel viral outgrowth tools MoltGGR and SupGGR. SupGGR cells are now being validated in a clinical setting by our collaborator Dr. Andrew Lever, University of Cambridge, prior to submission for publication.

four The fourth chapter applies knowledge from Chapters 2 and 3 toward capture, quantification, and characterization of primary HIV isolates in a clinical pipeline.

> The data described in Chapter 4 are being validated and

expanded through enrollment of additional patients into the patient cohort in Malawi. These findings will be submitted for publication within the coming year.

five The fifth and final chapter addresses the many questions raised by my research, and what I view as the future trajectory for these lines of inquiry.

CHAPTER ONE

Introduction to HIV replication biology and what it means for cells in the deep tissues

In this chapter I introduce the basics of HIV replication, the cell types it infects, and where these cells live. I describe the human airway as a compartment that may harbor HIV-infected reservoir cells, and present the alveolar macrophage as a compelling reservoir candidate. This chapter also discusses the current means to measure the HIV reservoir in human patients, including the strengths and relative weaknesses of these assays. The available literature and gaps in our knowledge frame the questions posed in the remainder of this dissertation and beyond.

Lifecycle of Human Immunodeficiency Virus

HIV is a positive sense, enveloped RNA retrovirus of the *Lentiviridae* genus[1,2], and the only lentivirus known to infect humans (HIV-1 and HIV-2). Mature virions are largely homogeneous, approximately 120nm in diameter, with a conical fullerene shell capsid that encloses two linear copies of the single-stranded RNA genome [3]; these two copies need not be identical in sequence, and may recombine prior to integration to drive genetic diversity [4]. As a retrovirus, HIV is defined by its RNA-dependent DNA polymerase "reverse transcriptase", a fascinating enzyme that reverses the central dogma of biology [5]. David Baltimore, and Howard Temin received the Nobel prize for their 1970 discovery of the enzyme and with it, founded the field of retrovirology [6,7].

HIV entry, reverse transcription and integration

Like all enveloped viruses, HIV gains entry to the cell by specific interaction of its envelope/spike protein with receptors on the cell, CD4 [8] and CCR5[9-13] (Fig. 1, reference[14]) CD4, a co-receptor of the CD3/T-cell receptor complex, is involved in interactions with antigen presenting cells during T-cell activation[15]. CCR5 is a chemokine receptor driving chemotaxis and activation of various immune cell subsets, including macrophages, osteoclasts and lymphocytes[15], though the receptor is expressed at much reduced levels in lymphoid cells[16]. Receptor binding is followed by an endocytosis-mediated pathway, where a membrane hemifusion event in early endosomes delays viral capsid ejection into the cytosol, a process that may or may not be pH dependent[17].

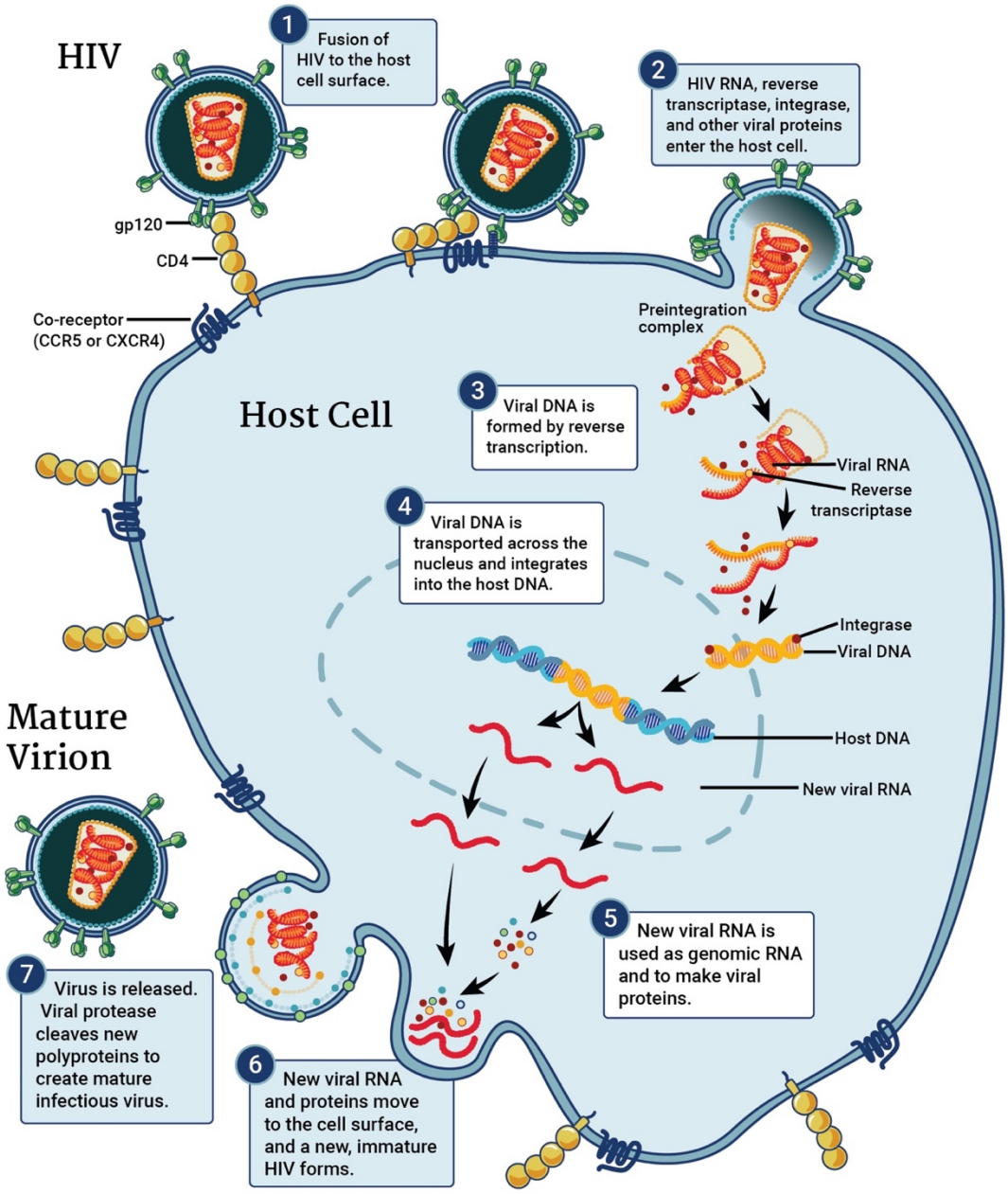


Figure 1. The HIV lifecycle. Access to the cell (**1**) is gained by binding of the envelope protein (gp120) to the cellular receptor CD4 and co-receptor CCR5, and conformational change, causing viral fusion. (**2**) the capsid is released into the cytoplasm, and is uncoated, releasing the viral genome and enzymes reverse transcriptase and integrase. (**3**) Proviral DNA is reverse transcribed and associates with the integrase/pre-integration complex for (**4**) transport into the nucleus and integration into the genome. Transcription factors drive HIV expression, and HIV-1 Tat amplifies this transcription (**5**). (**6**) Virions are assembled as structural proteins encapsidated the RNA genome. The virus is then released, causing maturation of the new capsid and resulting with infectious virus (**7**). SOURCE: NIAID

Release of the capsid into the cell is followed by uncoating of the RNA genome (Fig. 1,#2), and reverse transcription[18] (Fig.1, #3). Viral enzymes reverse transcriptase and integrase are packaged into virions and are functional upon release into the cytoplasm[18]. Importantly, cellular dNTP pools determine the rate of reverse transcription, and are modulated by the host restriction factor SAMHD1[19-23]; in non-replicating cells like macrophages, excess dNTPs are eliminated to interfere with viral polymerase activity. Production of proviral DNA from the RNA genomic template is completed in the cytosol, with the RNAseH activity of reverse transcriptase degrading the RNA template strand[18].

Proviral DNA then associates with the integrase enzyme and viral protein vpr (also packaged in the virion) into a pre-integration complex[24-26], a structure responsible for import of the provirus through the nuclear pore and integration of the DNA onto the host chromosome (Fig. 1, #4). Integration occurs at random, but is reported to bias toward euchromatin regions of the genome that are also close or tethered to the nuclear envelope[27].

Integration and transcription of the HIV provirus (Fig.1, #5) are dependent on the LTRs (long terminal repeat) found on both ends of the genomic RNA and integrated proviral DNA[28]. Sequence homology in both LTRs is required for proper topology of the provirus during integration[29], without which integration may fail or result in circularized LTRs[30].

Regulation of HIV transcription

Once integrated, the earliest HIV transcription events proceed in the absence of any viral proteins, relying solely on host transcriptional activators NF-kb/SP-1 or NFAT[28]. Baseline transcription in newly-infected cells drives inefficient production of HIV transcripts that are multiply spliced by the host machinery. The HIV genome encodes nine genes and several splicing variations that produce transcripts of three main sizes: ~1.8, 4.0, and 9.0 kb[28]. Four splice donor and seven acceptor sites govern the output from the primary transcript pool. The earliest translation product of HIV is the transcriptional co-activator Tat, and derives from the early multiply-spliced RNA class. Tat is a transcription factor unlike most, as it binds a sequence on the nascent RNA transcript and not the genomic DNA[31], significantly increasing elongation by recruitment of pTEF-b and associated factors[28,31].

Upon robust Tat-dependent transcription, other multiply-spliced, early genes are translated, notably Rev. As another RNA-binding protein, Rev binds with high affinity to the Rev response element (RRE), a region of RNA secondary structure within the HIV env ORF[28]. Rev binding to the HIV RNA results in additional Rev monomers oligomerizing, a process that increases the REV:RRE affinity by 500-fold[32]. These events drive rapid export of the message from the nucleus, dependent on the interaction of Rev with Crm1/ exportin-1 through the nuclear pore complex[33], delivering singly spliced and unspliced transcripts into the cytosol for translation. Importantly, the structural proteins of the gag-pol polyprotein and the env spike

protein are only translated during these later stages of transcription, dependent on Rev accumulation, and are characterized as 'late' genes of the HIV lifecycle[28].

Multiple higher order RNA structures on the HIV transcript govern its trafficking and encapsidation, most notably in the proximal region of the gag RNA[34,35]. Interestingly, the gag protein product binds its own coding transcript during the loading of genomic HIV RNA onto the nucleocapsid structure[36], an interesting wager by the virus on the importance of gag[37]. These features drive the strict sequence conservation of the gag region, both at the protein level and primary sequence level required for RNA secondary structure and protein binding.

Early establishment of HIV infection

Chronic HIV-infection in humans is characterized by a period of asymptomatic viral dissemination[38]. During this time, the virus spreads and adapts to a multitude of cellular reservoirs, including memory CD4-positive T-cell subsets, the definitive 'latent reservoir' for HIV in vivo[39-42]. The asymptomatic chronic phase is characterized by only a slow decline of CD4 cells in the mucosa and blood, coupled with persistent immune activation and gradually increasing viremia [43]. During this phase, humoral immunity suppresses any explosive replication, such as that observed during the acute phase of infection before seroconversion (the emergence of HIV antibodies in the blood). For patients on fully suppressive antiretroviral therapy (ART), new infections or reactivation of latent provirus will

produce virions that are then rendered non-infectious by drugs in the blood, or in the deep tissues where drug penetration reaches therapeutic concentrations [44].

The foundation of current ART regimens is the rapid arrest and death of HIV-infected T-cells, a process naturally mediated by the HIV protein Vpr [45], combined with the drug-based inactivation of any virions that are produced. Thus, after an initial period of viral decay following ART initiation [46] the number of HIV virions or HIV-infected/transcribing cells found in the bloodstream is vanishingly small. However, identification of these cells is a key challenge, since monitoring 'blips' or flare-ups of HIV infection during ART regimens is critical for the quantification of the size and temporal decay of the HIV reservoir with prolonged drug treatment [39].

Biphasic decay during ART reveals the HIV reservoir

Measurement of bloodborne HIV decay kinetics from patients newly started on ART therapy reveals a biphasic curve [46] similar to that in Figure 2. The two slopes of decay have been suggested to implicate two principal cell populations with different half-lives that continue to produce HIV after ART initiation, declining until HIV falls below the theoretical assay detection limit [41,43]. The interpretation of these results has been problematic for two reasons. First, with continued improvement of assay methodology, additional HIV replication activity below the theoretical detection limit has been proposed, suggesting HIV production is being 'missed' in standard assays[47,48]. Secondly, the shedding of HIV virions into the bloodstream

during ART must occur by rare cells in the blood or in tissues with ready access to the bloodstream. Thus, while our ability to detect bloodborne HIV has improved dramatically since the initial interpretation of the biphasic decay curves, an unmitigated obstacle remains: we still have no reliable means to relate the measured HIV burden in blood with the true HIV burden within tissue. More so, there is no data on the extent to which HIV-infected cells in tissues contribute directly to plasma viremia, if at all. In the same way that cerebrospinal fluid proteins or pulmonary surfactant proteins are not found in the bloodstream, tissue HIV virions are compartmentalized and do not cross endothelial boundaries passively[49,50]. During ART, HIV found in the blood is therefore a symptom of underlying tissue HIV infection; upon ART interruption or failure, these tissue reservoirs seed new infections in CD4 lymphocytes and other target cells that can actively enter the bloodstream to produce viremia[39]. Hence, the identification of HIV-infected cells during ART treatment remains a key paradigm in the search for curative HIV therapy. Ongoing work with humanized mice will likely soon provide some clarification of these issues [51], but these mouse models do not recapitulate the full spectrum of human HIV infection or persistence.

Myeloid reservoirs of HIV

Of considerable interest is whether these reservoirs can form in macrophages in addition to the well-described memory T-cell reservoir. Such a possibility is strengthened by the observation that HIV replicates and adapts within macrophages in late stage AIDS in humans when T-cell numbers dwindle [52], in T-cell

poor environments like the brain[53], and in humanized mice lacking CD4 T cells altogether [54,55]. In these scenarios macrophage-tropic virus strains emerge, indicative of underlying macrophage replication and adaptation over time. Macrophage-tropic viruses are able to infect cells with low expression of CD4 protein and high CCR5 expression, as found on a variety of myeloid cells in vivo and monocyte-derived macrophages and dendritic cells in vitro [53], but are also able to infect T-cells with high levels of CD4 and much lower CCR5 expression[56]. The reverse is not true, however: viruses highly adapted to infect T-lymphocytes (T-cell tropic) require very high levels of CD4 expression and are thought to infect macrophages with many fold lower efficiency[57]. Importantly, the key group of viruses called transmitted/founder (T/F) HIV are uniformly T-cell tropic using CCR5 as the co-receptor and high concentrations of CD4[57], and appear to have reduced entry and replication in human monocyte-derived macrophages (HMDM)[58]. These T/F viruses are identified within individual patients by careful population analyses[59]. In short, the sequence of the furthest common ancestor virus can be deduced from a pool of diverse strains found in a single patient by clustering related populations of viral genotypes. This ancestral genotype represents the closest estimate of the virus that infected the patient, and thus its properties including cell tropism, mechanism of transmission, and replication in various cell types are of intense interest. However, the likelihood that T/F viruses reside in tissue macrophages in vivo has been questioned, an argument supported by several key findings: (1) poor T/F virus replication over time in HMDM cultures in vitro[58], (2) inefficient entry of T/F envelope (env) proteins into cultured HMDM[60], (3) lack of available data showing

robust macrophage infection in human patients [61], and (4) the predominance of T-cell CCR5-tropic HIV at all stages of HIV infection in acute, chronic, or ART-treated HIV patients[62,63].

Taken together these observations seem to argue against a role for macrophages in harboring T/F HIV in vivo. However, they overlook key features of HIV biology in macrophages, and may underestimate the true ability of these complex cells to harbor T/F virus in vivo during persistence and transmission. First, these studies were performed on primary HMDM cultures, an arguably weak model for human tissue-resident macrophages. The classical monocyte-macrophage lineage was thought for many years to be the source of tissue-resident macrophages; over time, tissue macrophages were thought to be turned over and replaced by incoming blood monocytes that differentiate locally to macrophage cells[64,65]. Recently, however this paradigm has been revised. A series of critical and rigorous experiments demonstrated that tissue resident macrophages such as Kupffer cells (liver), microglia (brain) and alveolar macrophages (lung) derive from an embryonic yolk sac-fetal liver lineage that swarms the tissues in the days surrounding parturition[66-68]. Moreover, these studies showed the self-renewal of tissue macrophages by proliferation during normal homeostasis; thus, resident macrophages may persist for many years, even the life of the organism. Contrast this with HMDM, which differentiate from blood monocytes but are terminally differentiated and do not proliferate in vitro or in vivo[69]. Furthermore, HMDM are inflammatory effector cells, subject to polarization along the classical M1 and

alternative M2 activation pathways in the initiation and resolution of inflammation[69]. Conversely for example, alveolar macrophages (AMs) are largely anti-inflammatory cells, sentinels of the airway that discern true incoming pathogens from the continuous barrage of innocuous airborne stimuli that flood the airway with each breath[70,71]. In mammals, there is perhaps no greater evolutionary pressure than that of a maintained airway, and the function of AMs is to prevent non-specific inflammation in this compartment. Hence, AMs and HMDMs are highly divergent, yet HMDM are the exclusive human macrophage population used to model in vivo myeloid infection. On the basis of cell proliferation alone, a process disrupted by HIV, the HMDM model cannot represent proliferative tissue macrophage lineages. On a backdrop of the challenges to harvest macrophages from solid human tissues, and the scant information about optimal culture conditions ex vivo, the shortage of robust data on HIV in tissue macrophages is not surprising. However, their relevance to persistence and cellular transmission remains highly provocative, and recent findings of tissue macrophage embryonic origins has opened new opportunities to study these cells in the context of HIV infection.

CHAPTER TWO

Molecular identification of cellular HIV infection: Tools for functional genomics of HIV-infected cells in vivo

In this chapter, I describe the development of a fluorescent in situ hybridization assay to identify individual HIV-infected cells by flow cytometry. I discuss several ways the assay was refined to adapt it for improved sensitivity and specificity, and using the FISH:FLOW tool I present important caveats for the use of a standard HIV reagent.

> The section in chapter 2 on Quantitative HIV biology with FISH:FLOW population analyses is published as a corresponding author paper:
>
> Wilburn KM, Mwandumba HC, Jambo KC, Boliar, Solouki, Russell DG, **Gludish DW**. *Heterogeneous loss of HIV transcription and proviral DNA from 8E5/LAV lymphoblastic leukemia cells revealed by RNA FISH:FLOW analyses.* Retrovirology. 2016 Aug 11;13(1):55

The experiments described in this chapter were performed in co-operation with two rotation students in the laboratory, Kaley Wilburn (FISH protocol optimization with zwittergent and probe titrations, and 8E5 subcloning and FISH:FLOW characterization) and Sabrina Solouki (8E5 p24/FISH co-labeling).

INTRODUCTION

Cell-level identification of HIV in vivo

Identification of HIV-infected cells in vivo using current technology is challenging[72]. While the p24 antigen assay (measure of HIV gag production by flow cytometry or ELISA) is an industry standard approach for research purposes, background staining and the small yield of HIV-positive cells in even large volumes of patient blood makes this approach prone to artifacts; the theoretical detection limit of these assays is 0.1%[72], which in many cases far exceeds the proposed HIV-infected fraction in the blood during ART (0.5-10 cells per million)[72,73]. Molecular identification of HIV by protein antigen staining alone is highly problematic, and must be considered a very minimal estimate of the true cell-associated HIV burden. Furthermore, recent movements in the field suggest that targeting HIV in tissues – not just in the blood – will be a necessary step to clear HIV reservoirs that persist during ART[39]. A streamlined approach to identify HIV-positive cells from patient tissue material is needed to understand where to find HIV reservoir cells, to quantify their decay in the face of current ART regimens, and to learn how to target them for destruction with future therapies.

To this end, we pursued a novel molecular assay to detect HIV-positive cells in a bulk population. Using a fluorescent in situ hybridization approach (FISH), individually fluor-conjugated, single stranded DNA oligonucleotide probes are hybridized to cell-associated HIV-1 RNA in a cell suspension (Fig. 2). After washing, cells are analyzed by flow cytometry (FLOW) or by fluorescence-associated cell

sorting (FACS) to detect HIV-1 probes, even in rare cells. Importantly, many short (~25bp) probes cover a long section of the target HIV RNA, enabling more tolerance of mismatches in the genetic sequence than would be accommodated during antibody staining. We present a workflow for identification of prospective probe targets, and highlight the flexibility of the FISH:FLOW platform in multiple cell systems.

FISH:FLOW optimization: toward RNAseq on sorted cells

Our laboratory previously developed the HIV FISH:FLOW assay using probesets targeting HIV-1 gag[74] (Fig. 2). The discovery of HIV-positive cells among AMs in human BAL was a first, important step towards characterizing this tissue compartment as an anatomical HIV reservoir. However, several key points remained unresolved. For example, does the RNA signal observed correlate to productive HIV infection? The FISH:FLOW method cannot distinguish full length HIV from highly deficient proviral insertions that are now thought to predominate in vivo[75,76]. Similarly, does the HIV RNA observed give rise to replication competent virions that could initiate infection in new host cells? To qualify as reservoirs, HIV-positive AMs must shed new infectious virus capable of initiating rebound infection after ART treatment is interrupted.

Definitive answers to these questions require at least one of two lines of evidence. Either the complete sequence from individual infected cells must be determined and the full-length virus synthesized for testing replication competence,

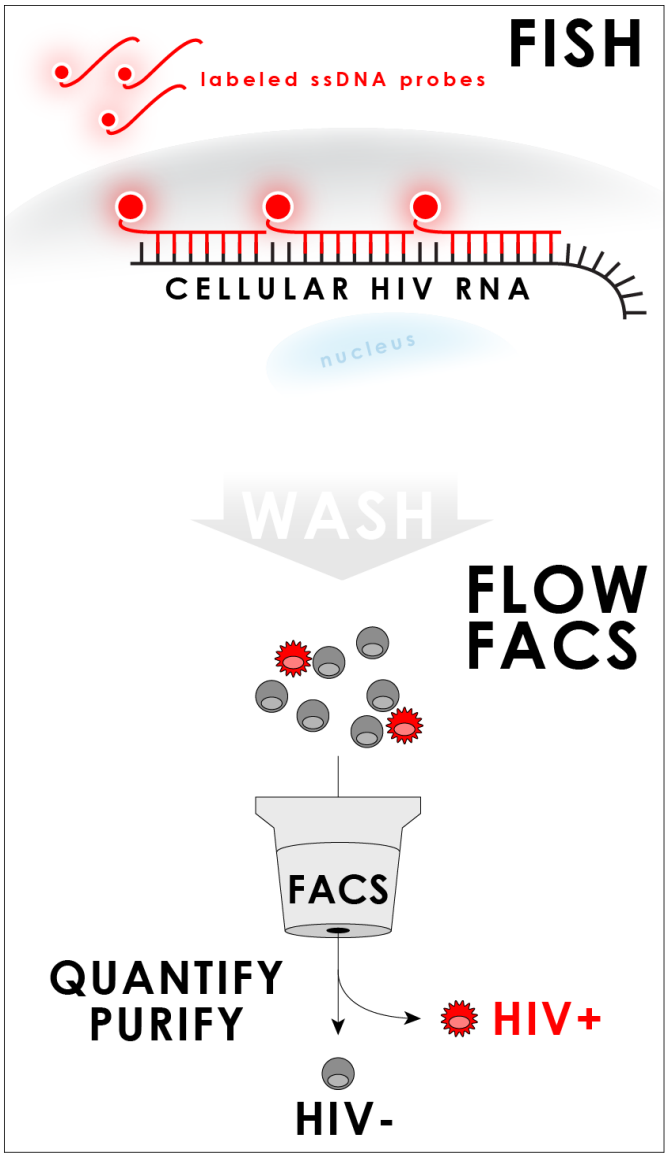


Figure 2. Schematic of FISH:FLOW pipeline. A series of short (~25nt) single stranded (ss)DNA probes each with a fluorescent label are hybridized to RNA within PFA-fixed and ethanol-permeabilized cells in suspension. Approximately 1000bp of contiguous HIV mRNA is detected with a given probeset. After washing the cells are passed through a flow cytometer to quantify increased HIV signal in the infected subset (red cells). Using a fluorescence activated cell sorter (FACS), the HIV probe-positive and -negative cell populations can be purified for transcriptional profiling and functional genomics.

or the primary AMs must be cultured with susceptible cells to establish supernatants with replicating virus. Ultimately the demonstration of infectivity must include some measure of virus outgrowth in vitro or in vivo[48,77-80]. In complimentary approaches, we pursued both the sequence-based and outgrowth-based platforms to characterize the potential HIV reservoir within AMs of the human lung.

RESULTS

Adaptation of the FISH:FLOW platform for purification of HIV-infected cells

The primary objective of these early experiments was to identify the viral genetic sequence within HIV-infected AMs isolated from Malawian adults by BAL. By staining BAL cells with FISH probes and physically sorting the positive cells by FACS, enrichment of viral RNA in the cell population would enable PCR- or RNAseq-based derivation of viral sequences. A key strength of the RNAseq pipeline is the potential to discern multiple sequence variants, and thereby examine the heterogeneity of the virus in the airway compartment during various phases of HIV infection. In theory, one could determine the types of mutations/variants accumulating in specific cell populations (AM vs. lymphocyte) in the airway in the presence or absence of ART therapy, or during acute or chronic infection. These are highly compelling opportunities, given the paucity of robust clinical data on cellular reservoirs and viral persistence among Clade C patient cohorts in Africa.

Based on our lab's prior success using FISH:FLOW staining in human AMs[74], I planned a pilot experiment to demonstrate proof of principle in human-derived material using PBMC from a chronic phase HIV patient not on ART. PBMC were used

in this experiment given their ease of access from all patients attending the HIV clinic at the Queen Elizabeth Hospital in Blantyre, Malawi. Bulk PBMC were stained with HIV *gag* FISH probes and prospective HIV-positive cells were FACS-purified on a BioRad S3e instrument (Patient No.272, Fig.3A). RNEasy FFPE-isolated RNA was prepared for RNAseq and the sequence analysis performed as described in Methods.

Alignment of the RNA reads to the HIV genome illustrated what is perhaps not surprising in retrospect. The greatest abundance of HIV reads aligned with the LTR sequences: areas of the genome present on every transcript off the provirus (Fig. 3). Conversely, the V3 ectodomain loop region of env had the lowest coverage, suggesting the genetic divergence of the input virus in this region precluded correct alignment with the NL4-3 reference genome. Confidence in the transcription data is increased by the observation that the aligned reads begin just

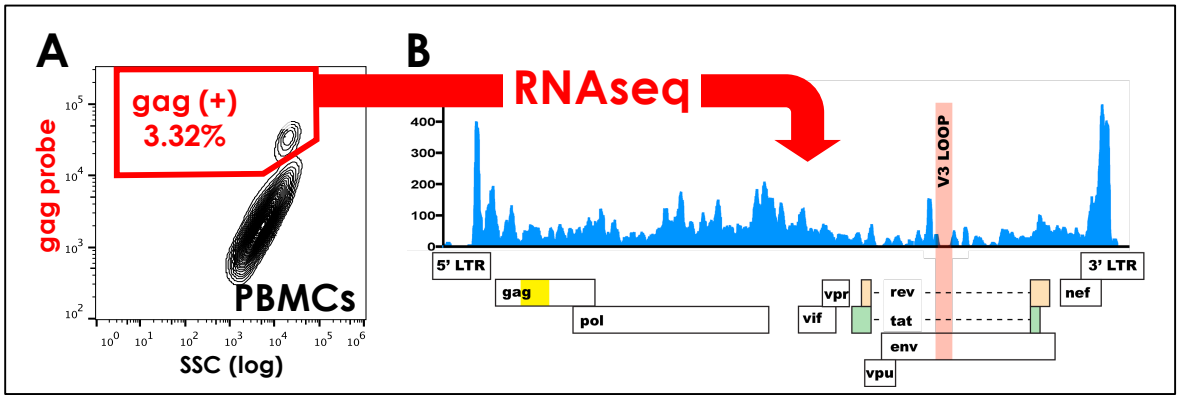


Figure 3. HIV transcriptomics of HIV-infected PBMC by FISH:FACS purification. A Bulk PBMC from patient No.272 were stained with HIV gag FISH probes and purified on a BioRad S3e instrument. Isolated RNA was sequenced on the MySeq instrument as described in Methods and **B** reads were aligned to the NL4-3 reference genome using Geneious 8. The area in the gag ORF targeted by FISH probes is highlighted in yellow. The V3 loop is highlighted in pink, and represents an area of high sequence variability where the reads returned failed to align with the reference genome.

downstream of the annotated transcription start site in the distal 5' LTR TAR region, suggesting a transcription product, and not contaminating plasmid or genomic DNA, is the source of the aligned reads.

While the fluorescence shift of *gag*-positive cells in the PBMC cell population was discernable, somewhat limited separation was observed between positive signal and background staining (Fig. 3A). I hypothesized that multi-color staining or improved probe design might also improve gating for future sorts, especially from primary human tissues where transcription of HIV might be lower than in peripheral lymphocytes. Human AMs in particular are variably auto-fluorescent, thus robust detection reagents will aid identification of the HIV-positive and -negative cell fractions in BAL samples.

Development of improved FISH probes (NEF)

Informed by the coverage distribution of RNAseq reads above (Fig. 3B), I reasoned that FISH probes targeting the LTR or nef regions might yield improved assay performance. To test this, additional probesets were designed against the 5'LTR region, the 3'LTR (including some nef ORF) and the nef-exclusive region (Fig. 3). These sequences are present in every transcription product of the inserted proviral genome and thus may represent more optimal detection targets. The probes were tested in JC-53 HeLa cells (stably express HIV co-receptors CXCR4, CCR5, and CD4)[81] infected 48 hours prior with VSV/G-pseudotyped HIV-GFP (Fig. 4A). Relative to mock-infected cells (Fig. 4B-G), comparison was made between gag probe staining and each of the new probes (Fig. 4H-M). Interestingly, versus the

gag probeset (Fig. 4I, orange) a more dramatic shift was observed using the probes to *nef* (Fig. 4JK, purple) and 3'LTR (Fig. 4L, magenta), while the 5'LTR (Fig. 4M, blue) probe generated an intermediate shift. The separation of these populations is better

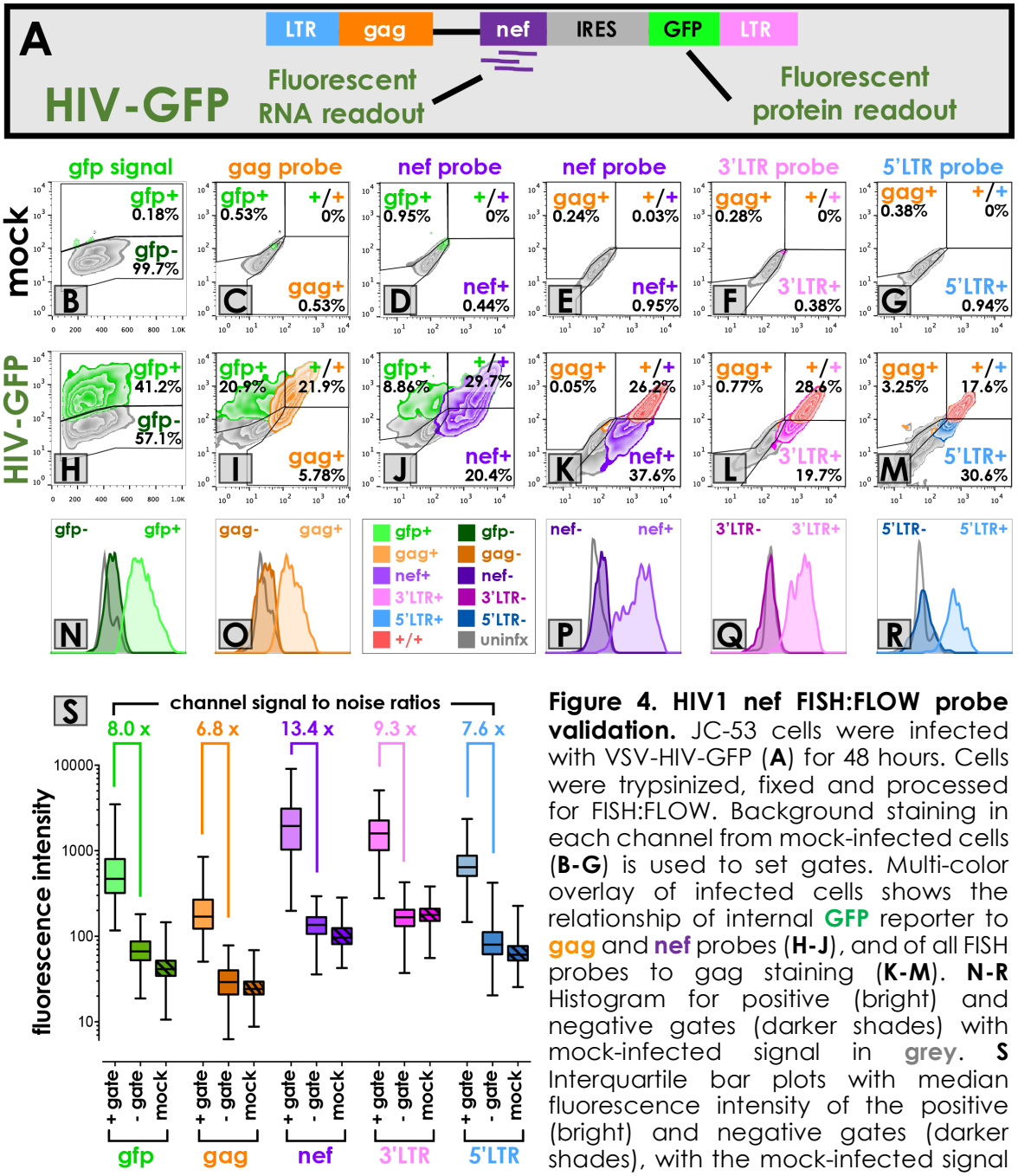


Figure 4. HIV1 nef FISH:FLOW probe validation. JC-53 cells were infected with VSV-HIV-GFP (**A**) for 48 hours. Cells were trypsinized, fixed and processed for FISH:FLOW. Background staining in each channel from mock-infected cells (**B-G**) is used to set gates. Multi-color overlay of infected cells shows the relationship of internal **GFP** reporter to **gag** and **nef** probes (**H-J**), and of all FISH probes to gag staining (**K-M**). **N-R** Histogram for positive (bright) and negative gates (darker shades) with mock-infected signal in **grey**. **S** Interquartile bar plots with median fluorescence intensity of the positive (bright) and negative gates (darker shades), with the mock-infected signal for each channel (hatched). The calculated signal to noise ratio (S/N) for each channel is given.

appreciated in the fluorescence histograms, Fig. 4N-4R. Importantly, these probes yielded improved signal to noise ratios over gag FISH staining (gag S:N 6.8x, Fig. 4S orange). Indeed, the signal to noise ratio of 13.6-fold for the nef probe (Fig. 4S purple) was a twofold increase over the ratio of gag probe, and even gave greater signal separation than internal GFP reporter carried by the virus (8.0-fold S:N, Fig. 4S, green). For each channel, the median fluorescence intensity (MdFI) for the positive and negative gates in infected and FISH-stained cells (colored bars in Fig. 4S) were compared with the MdFI of uninfected cells stained in the same experiment (hatched bars, "mock", Fig. 4S). Uninfected (FISH-negative) bystander cells and the bulk cells from uninfected wells exhibited similar staining distributions, suggesting cells from infected wells that stain positive for FISH probes harbor true HIV transcription, even in a mixed population of high HIV penetrance.

A key observation was made that for *nef* and 3'LTR probe staining, almost no *gag* signal was observed in the *nef*-negative (0.05%, Fig. 4K gag+/nef-) or 3'LTR-negative gate (0.77%, Fig. 4L gag+/3'LTR-). This finding is consistent with the well-described staged transcription of HIV and the 'late' character of gag gene transcription[28]; no gag transcription is expected in any cell without early transcripts such as nef first expressed. Together, the data demonstrate (1) that additional FISH probes to these high-abundance transcript regions might improve the separation of HIV probe-positive cells in clinical samples, and importantly that (2) FISH:FLOW provides a means to dissect the lifecycle of HIV *in vivo,* discriminating early from late gene transcription at the level of individual cells. This is of particular interest given

the shortage of methods to conveniently quantify the reactivation of latent HIV during 'shock and kill' approaches to target the HIV reservoir in human clinical trials[39]; the effects of such strategies on the size of the viral reservoir remain difficult to quantify.

Importantly during the course of this work, similar RNA FISH-based approaches to reservoir quantitation were published[72,73], reflecting heightened interest in cell-level readouts during HIV reservoir analyses. The authors focused on a mixed protein/RNA FISH staining platform both tied to the gag/pol ORF, and thus lack resolution to distinguish early (new) from late (established) infections. In relevant tissue reservoirs like the human airway, it is of great interest to learn whether established infection in AMs persist during ART, or whether evidence of new infection can be found. The FISH:FLOW method with physical purification is ideally suited to enrich HIV-infected cells from a bulk lavage population, simultaneously learn whether any of these infections are likely to be recently-infected cells, and to characterize the transcriptional profile of the infected host cells. This last point is critical to specifically target HIV-infected cells in the tissues. A recent report made a similar advance for peripheral blood reservoirs, and identified CD32a as a specific surface marker of T-lymphocytes harboring latent HIV proviruses[82].

Establishment of the FISH:FLOW assay for Simian Immunodeficiency Virus (SIV)

Controlled *in vivo* studies of the HIV latent reservoir are confined to model systems like macaque monkeys with SIV or SHIV hybrid viruses. Any broad adoption of the FISH:FLOW platform requires portability to the related but not identical SIV

model system. To establish the utility of the FISH assay in this arena, new *gag* and *nef* probes were manufactured to target SIVmac239, a common reference virus used during SIV studies *in vivo*[83]. VERO cells (from African green monkey kidney) were infected for 48 hours with viral supernatants containing SIVmac239, then trypsinized, PFA-fixed and stained with SIV FISH probes as described above for HIV *in vitro* infections.

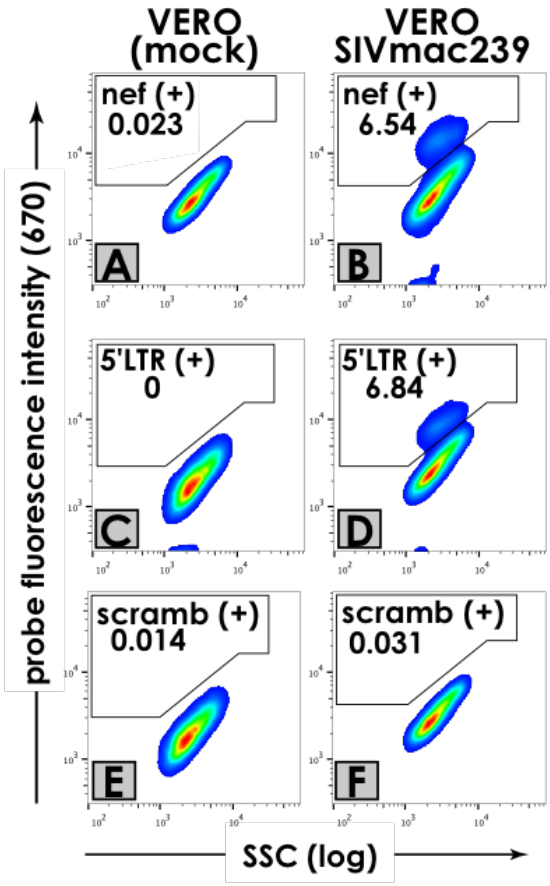


Figure 5. SIVmac239 nef FISH:FLOW probe validation. VERO cells were infected with SIVmac239 for 48 hours. Cells were trypsinized, fixed and processed for FISH:FLOW. Background staining for each probe in mock-infected cells (**A**,**C**,**E**) was used to set gates. Infected cells (**B**,**D**,**F**) stain positive for FISH probes to nef (**B**) and 5'LTR (**D**), but not with an irrelevant probe (scramb, **F**).

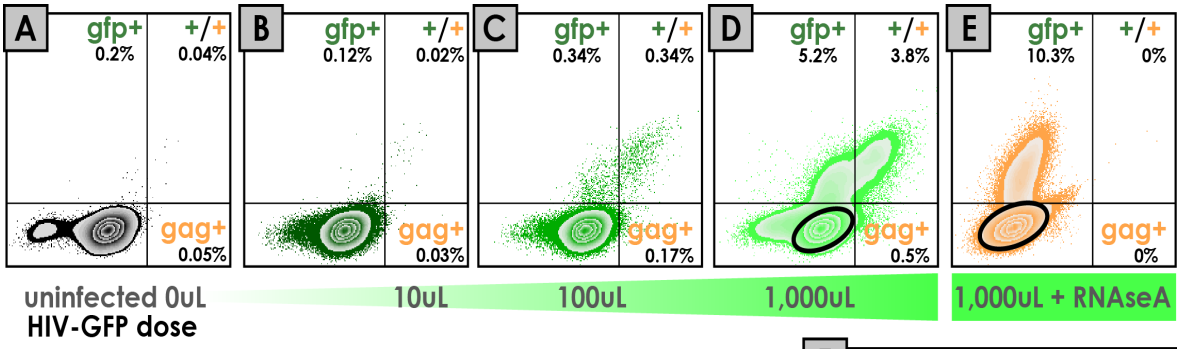
FISH:FLOW analyses revealed that cultured VERO cells generated clean SIV *nef*-positive signal compared to uninfected control cells and, importantly, to cells stained with an irrelevant/scrambled, fluor-labeled probe (Fig. 5). However, the shift in SIV *nef* fluorescence was less robust than that observed during parallel experiments staining *in vitro* infected JC-53 cells with the HIV nef probe (Fig. 6). While VERO cells may be less ideal hosts for SIV transcription, the results suggested additional optimization might improve the dynamic range of the assay. After taking stock of the FISH:FLOW assay strengths and weaknesses, considerable investment was made to address some systematic issues, and to improve the specificity and sensitivity of the FISH:FLOW assay, summarized below.

TABLE 1. Optimization of the RNA FISH:FLOW assay

Technical issue	Remedial action/troubleshooting	Data
Weak shift with *gag* probe	New probe design: *nef*	Fig. 4
Cell loss during liquid transfer	Detergent/ Denhardt's Solution	Not shown
Uncertain probe target	RNAseA treatment post-fixation	Fig. 6
Low RNA quality post-fixation	OneStep fixative, RNAse inhibitor	Not shown
Low signal:noise ratio	Blocking reagent optimization	Fig. 7
Single criterion (RNA) detection	Multiplex with antigen staining	Fig. 11

Determination of FISH:FLOW probe target specificity

It is important to determine whether the signal observed during FISH:FLOW analyses corresponds to HIV RNA, and not genomic DNA or other non-specific binding. To address this, JC-53 cells were infected with a titration series of HIV-GFP (Fig. 6), and monitored for GFP expression. At 36 hours post-infection, the cells were trypsinized, fixed in PFA and treated with RNAse A (or 2X SSC vehicle control) prior to processing for FISH:FLOW analyses with *gag* probes (Fig. 6). Thirty-six hours after inoculation with the highest dose (1mL) of virus supernatant, approximately 9% of analyzed cells were positive for GFP expression compared to mock-infected controls, with less than half of those (3.8% total) also staining *gag*-positive by FISH. This result highlights the nature of the GFP reporter in the HIV-GFP construct, where the IRES-GFP cassette is regulated with the nef ORF (Fig. 4A), an early Rev-



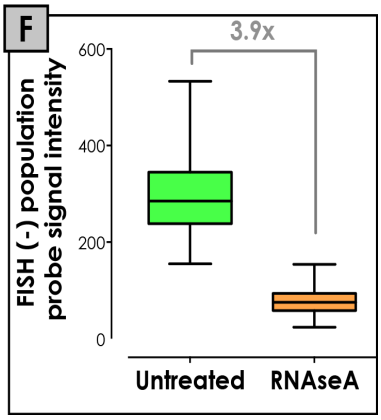


Figure 6. FISH probes detect mRNA in HIV-infected JC53 cells. A-D Titration of HIV-GFP inoculum dosage yields increasing fractions of GFP-positive JC-53 cells. Gates were set using uninfected cells (**A**). Replicate cultures of the highest HIV dosage (1,000uL, **D**) were treated with 100µg/mL RNAseA for 20 minutes at 37°C in 2X SSC buffer prior to washing and processing for RNA FISH:FLOW. To analyze shifts with and without RNAse treatment, the elliptical gates in **D** and **E** are plotted in **F**. The median fluorescence intensity (black line, **F**), interquartile range (colored bars), and the 1-99 percentile error bars demonstrating signal range are shown.

independent gene, while *gag* is a late gene requiring production of Rev. Thus, the GFP-positive but *gag*-negative cells represent a population of recently-infected cells in the early phase of viral transcription – this is an expected finding at this early timepoint post-infection (36 hours).

Importantly, treatment of the cells with RNAseA prior to FISH hybridization revealed two key insights. First, loss of total cellular RNA completely ablated the *gag* FISH signal (note the GFP fluorescence signal was spared), showing the species detected by HIV FISH is in fact RNA. And secondly, a nearly fourfold decrease in FISH probe signal intensity was observed in the HIV-negative cell population upon treatment with RNAseA (Fig. 6, 3.9x MFI decrease), suggesting that cellular RNA in general serves as a site for non-specific probe binding. While the FISH protocol includes E.coli T-RNA as a blocking reagent in the hybridization buffer, the observation that non-specific signal was ablated upon digestion of cellular RNA suggested that a strategy to block the binding of fluorescent probes to cellular mRNA would enhance the specificity of the assay.

Optimization of FISH:FLOW hybridization conditions

I devised a modified blocking strategy to (1) compete with non-specific binding of ssDNA probes and (2) provide more stringent interactions of probe with target. The first aspect was approached with a scrambled, unlabeled ssDNA oligonucleotide, more akin to the actual target FISH probes used for staining than the highly ordered tRNA reagent used in the existing FISH protocol. The second

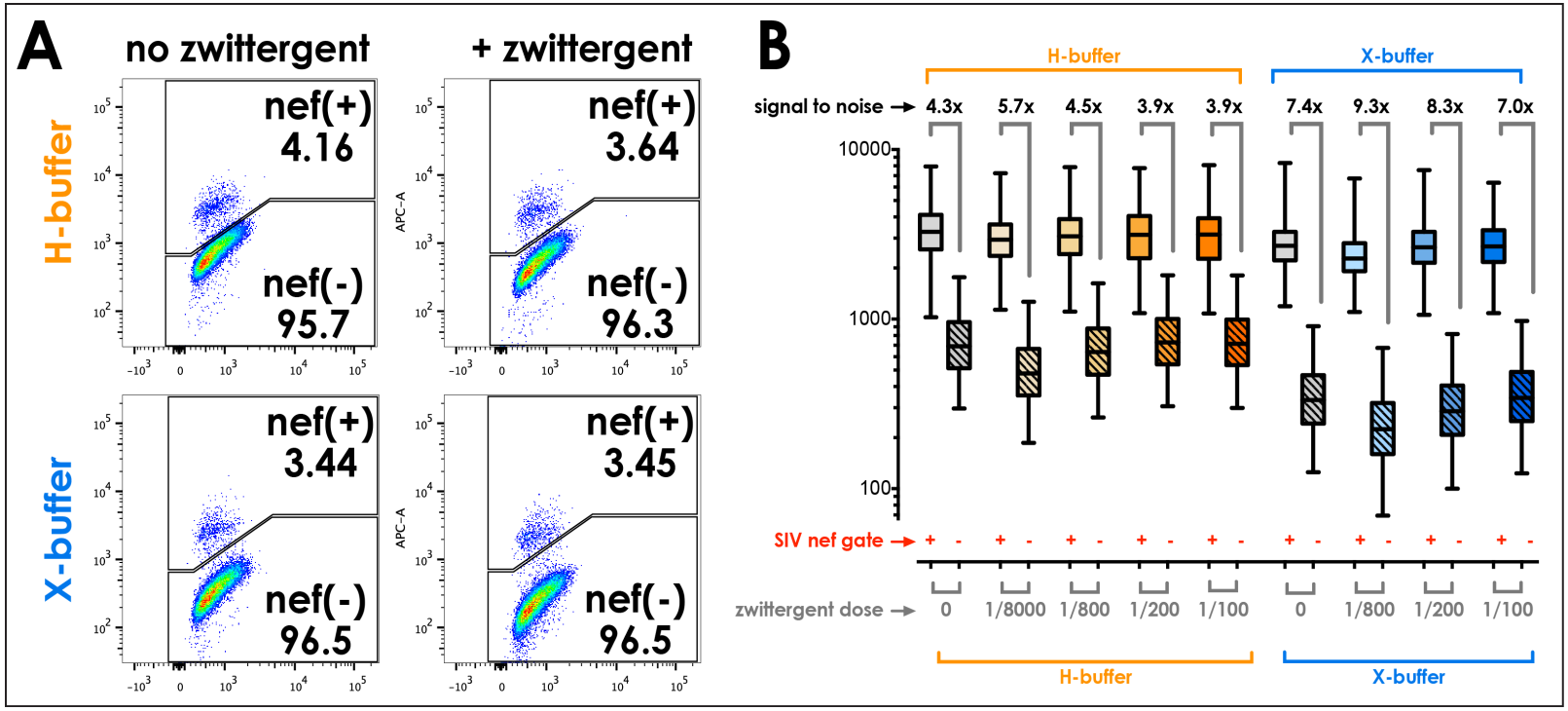


Figure 7. A Zwittergent hybridization additive enhances signal separation and augments the activity of an unlabeled oligonucleotide blocking reagent. A SIVmac239-infected VERO cells were stained with RNA FISH probe to SIV nef, and analyzed by flow cytometry. Hybridization reactions were carried out in standard H-buffer (orange) or X-buffer (blue) containing unlabeled oligo blocking reagent. Reactions were performed with and without Zwittergent 3-12 in a dilution curve, as indicated. **B** Median and interquartile fluorescence plots of the SIV nef-positive(colored boxes) and-negative gates (corresponding hatched boxes), showing an inverse correlation of signal-to-noise ratio with zwittergent dose.

parameter of stringency was addressed by titrating a detergent, Zwittergent 3-12, into the hybridization reactions. Hybridization reactions were carried out using various concentrations of zwittergent and equal concentrations of either E.coli tRNA ("H-buffer") or the new dsDNA oligo blocking reagent ("X-buffer"), and the hybridization carried out as previously. The performance of the blocking reagent was established in SIV-infected VERO cells, given the relatively weaker signal-to-noise ratio in this system.

The SIV *nef*-positive signal obtained using X-buffer hybridizations was modestly lower (~15%) compared to the standard H-buffer sample (geometric mean calculations, Fig. 7B). However, a much larger decrease of more than 50% was observed in the SIV-negative gate, indicating the oligo blocking reagent improved the separation of the negative and positive cell populations in the FISH assay. This was quantified as the mean signal to noise ratio, dividing the geometric means of the positive and negative gates (Fig. 7, ratios indicated above each group).

Interestingly, signal improvements were also gained using Zwittergent addition, but the effect observed was inversely correlated with zwittergent dosage. While zwittergent addition routinely provided improved signal to noise metrics compared to no detergent added, the most dilute preparations of zwittergent yielded the best signal separation (Fig. 7AB). Using standard hybridization conditions (H-buffer, no zwittergent), the signal to noise ratio for the SIV probes was 4.3-fold,

while the best condition tested was oligo/X-buffer hybridization (9.3-fold) with the lowest concentration of Zwittergent tested in this experiment (0.00125 mg/mL). These results were repeated in several experiments with similar results, revealing that Zwittergent performance was optimal with low concentrations of FISH probe in oligo-containing X-buffer (unpublished data).

The inverse dose response of Zwittergent could be due to several factors, including potential autofluorescence of the detergent, or its induction of autofluorescence among fixed cells or nucleic acids within FISH-stained cells at higher detergent concentrations. In this scenario, any effects of lowering background probe binding would be counteracted by higher autofluorescence. However, acquired signal in the unstained FTIC channel was not affected at any zwittergent concentration, arguing the improved hybridization signal to noise ratio in the presence of zwittergent was related to the probe:target or probe:non-specific target interaction, and not to fluorescence of the zwittergent reagent itself. Future experiments should test this using titrations of a labeled, scrambled/irrelevant probe, and confirm the effects in primary cultured macrophages prior to its use in human clinical samples. However, the reproducible effect of zwittergent in these reactions suggests this additive is a useful tool to gate FISH-positive cells during flow cytometry.

Quantitative HIV biology with FISH:FLOW population analyses

It is well established that HIV establishes a latent reservoir in memory CD4+ T-

cell subsets[39,40], rare cells that exit the cell cycle following activation and HIV infection. In the absence of activation-associated genes such as NF-kB, HIV transcription is ceased and the provirus enters 'latency'. The infected cell circulates until a time when the cell encounters specific antigen, or is otherwise activated into proliferation. This latent reservoir is the roadblock to an HIV cure, since latent virus is not immunogenic, produces no cytopathic effects, and is not cleared by the immune system[39]. 'Shock and kill' is a recently proposed strategy to achieve functional HIV cure, and involves screening for chemical compounds or other biomodulators that can drive HIV proviral transcription in latent cells[84]. This strategy aims to avoid wholesale activation of all memory cells, a scenario leading to hyperactivation and a likely fatal cytokine storm. While some progress has been made toward this goal, it became clear early on that we lacked a robust assay to measure the HIV transcription at the cellular level to learn whether prospective treatments were having the desired effects in vivo. Whole sessions at several recent high-profile HIV meetings were dedicated to such assays. A suitable approach would also directly quantify the inducible reservoir, and thereby help illuminate the scale of the challenge faced in pursuit of an HIV cure. Currently, the field has several means to measure the inducible HIV reservoir, and they all seem to give different answers and make different assumptions about the underlying cell biology[77,79,80,85]. Until very recently, cell-level readouts were not available for such studies.

The FISH:FLOW assay would seem an ideal tool for quantitation of HIV-transcribing cell populations. While the assay requires the provirus to be actively

transcribing (it is an RNA assay), and is therefore not useful in measuring the latent, resting HIV reservoir, I reasoned that any approach to shock cells out of latency could be easily quantified with FISH staining. In an effort to establish the quantitative sensitivity of the assay using a cell platform in common use among HIV researchers, I turned to the 8E5 cell line[86].

8E5 lymphoblastic leukemia cells

The 8E5 subclone was isolated from HIV(LAV)-positive A3.01 lymphoblastic leukemia cells that survived HIV infection *in vitro*[86], and was the first such reagent described in the literature. Early analyses revealed that all 8E5 cells harbored a single HIV-1/LAV provirus, shedding intact but defective HIV virions[86]. A single amino acid substitution in the reverse transcriptase (RT) coding region of the gag-pol polyprotein renders the virions non-infectious[87], effectively holding static the 100% HIV penetrance among 8E5 cells in culture. It was not established why this particular cell clone was refractory to the cytopathic effects of the virus, but a lesion in the cell cycle arrest machinery required by the HIV gene *vpr* seems a likely candidate. As a result of their properties, these cells have been employed worldwide for the standardization of patient sample PCRs in the clinic[88], and are ideally suited as tools for validating single cell approaches to study the latent HIV reservoir. Using serial dilutions of 8E5 cells with known proportions of uninfected lymphoblasts, a standard curve can be generated using cell associated RNA or DNA – the same input material that can follow an identical sample pipeline as the patient-derived material being tested by PCR or other approaches.

Unexpected low penetrance of HIV mRNA expression in 8E5 cultures

To test the limits of the FISH:FLOW assay, I generated a dilution series of 8E5/CEM cell standards and analyzed them by FISH:FLOW. Surprisingly, I found the starting 8E5 population was heterogeneous with only 4.5% positive for HIV *nef* (Fig. 8A) and 2.9% for *gag* transcripts detected by RNA FISH (Figure 8B). However, almost all gag-positive cells also stained nef-positive as expected (Fig. 8C). Dilutions of 8E5 cells with uninfected CEM cells yielded a linear standard plot (Fig. 8D), as might also be expected from PCR analysis of comparable dilution series. However, the absolute number of viral genomes inferred by this method would underestimate the true values obtained using standards that assumed 100% of 8E5 cells in the starting population were HIV-infected.

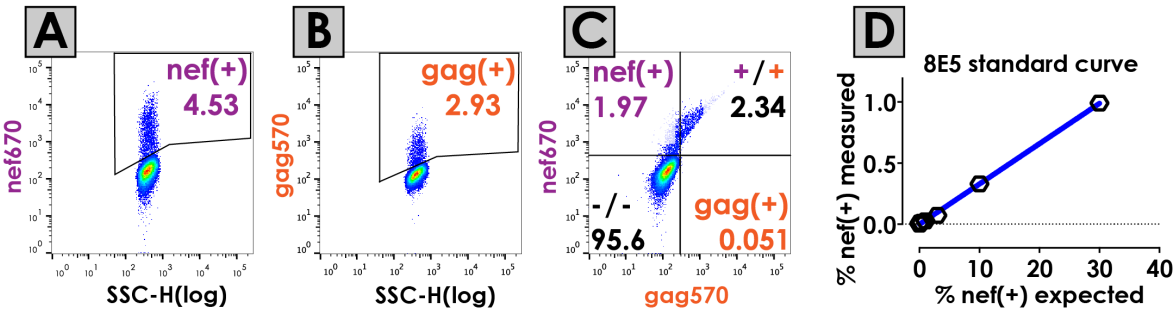


Figure 8. 8E5 cells exhibit partial HIV mRNA penetrance but yield linear standard curves in dilution series. A,B 8E5 cells stained with HIV nef FISH probe (Quasar670, **A**) or HIV gag570 probe (**B**), revealing low penetrance of HIV transcription in the 8E5 population. **C** Multiplex FISH stain, showing that all gag-positive cells are also HIV nef-positive. **D** Sub-penetrant 8E5 cells were mixed in known proportions with uninfected CEM cells to generate a standard curve. Populations were stained by FISH and quantified for HIV penetrance by flow cytometry (not shown). The measured % nef-positive population was plotted against the expected % nef-positive fraction, according to a fully penetrant 8E5 population. A linear titration curve is obtained despite the actual penetrance of 8E5 of less than 5% in this population, revealing the inherent weakness of the 8E5 calibration standard for HIV genome quantification.

Maintenance of HIV expression in a cloned subpopulation of 8E5 cells

HIV transcription may not proceed in all 8E5 cells at all points in the cell cycle, a scenario that could explain a subpopulation of HIV RNA-negative cells in 8E5 cultures. However, given the observed low fraction of positive cells, I reasoned that proviral loss or durable silencing[89,90] would more likely account for a majority of cells negative for HIV *nef* or *gag* mRNA. To assess the maintenance of the HIV proviral genome in 8E5 cells 200 subclones were generated by limiting dilution, and expanded for analyses by FISH:FLOW (Fig. 9). Subclones were first combined into pools of 20, and screened for HIV positivity. Surprisingly, only one subclone pool (Pool J) showed signal for HIV *nef* at a frequency suggesting that only a single clone in the pool was positive (Figure 9B). When subclones were analyzed individually it was found that most clones were entirely negative for *nef* RNA (representative clone J3, Figure 9B). By contrast, clone J20 was homogeneously positive for HIV *nef*

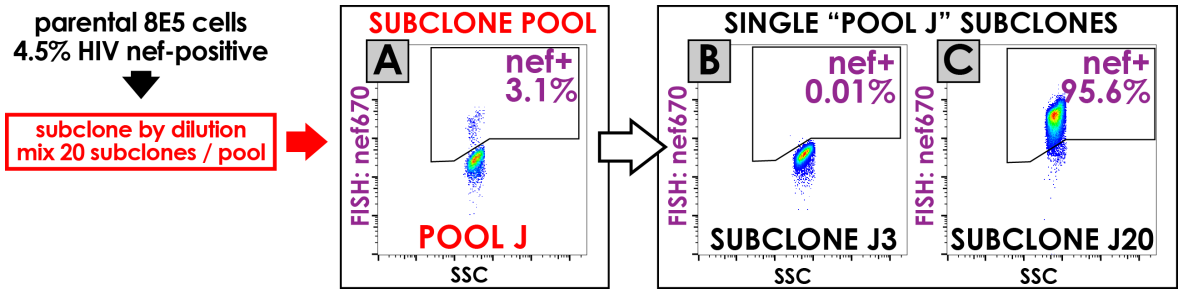


Figure 9. HIV FISH analyses of 8E5 subclones yields a single HIV-positive clone, J20. The sub-penetrant 8E5 population was subcloned by limiting dilutions in multiwell plates. Individual clones were picked and pooled into groups of 20 subclones to screen for HIV-positive pools. Pool J (**A**) was the only pool staining positive for nef. Analyses of individual "J" subclones (**B,C**) reveals nineteen subclones are HIV negative (representative clone J3, **B**), and a single subclone "J20" (**C**) is fully penetrant for HIV nef transcription.

RNA by FISH:FLOW analyses (Figure 9C). FISH:FLOW analysis with HIV *gag* RNA probes yielded similar results (data not shown). Interestingly, the lower than expected frequency of HIV-transcribing clones (1/200 vs. ~4.5/100 expected) suggests that cells containing active HIV proviral genomes are at a survival or clonogenic disadvantage compared to those that have silenced or lost the provirus.

Absence of HIV transcription could result from proviral silencing or loss of proviral genomic DNA. Either scenario could be the product of negative selective pressure experienced by HIV-infected lymphoblasts in long-term culture. Transcriptionally silent or HIV-negative subclones within the 8E5 population would have a growth advantage, and would rapidly outcompete the HIV-expressing population. To address this experimentally I compared relative frequencies of

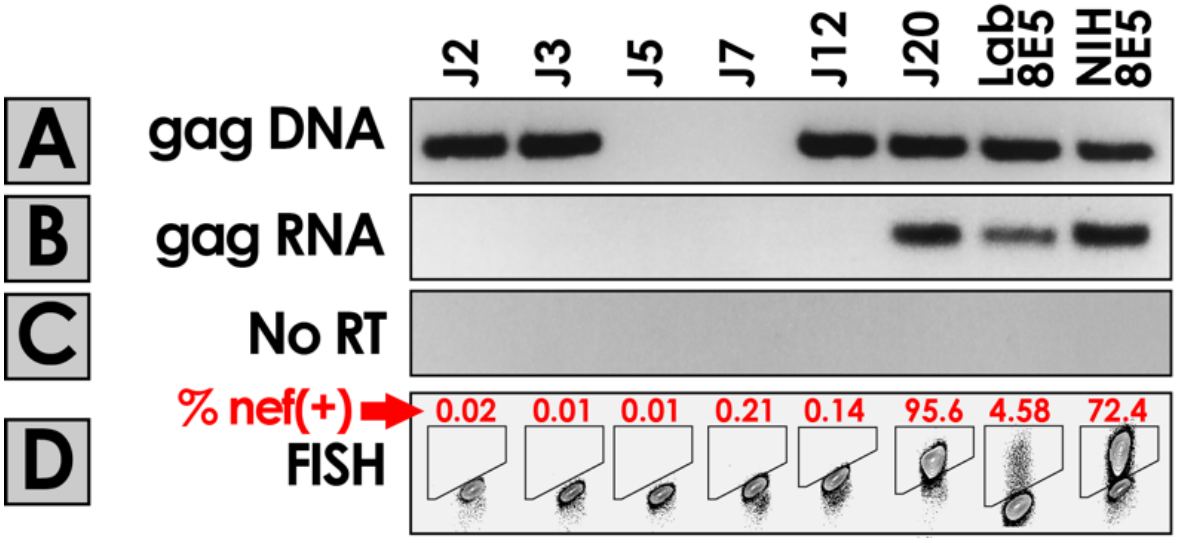


Figure 10. 8E5 subclones exhibit proviral silencing and DNA loss. A HIV gag DNA PCR identifies pool J subclones harboring provirus. **B** Corresponding gag qRT-PCR products reveals only clone J20 expresses gag mRNA. **C** Amplification of cellular RNA samples with no RT added. **D** Corresponding nef FISH plots reveal that HIV nef FISH:FLOW predicts gag PCR outcome.

proviral DNA (Qiagen Qlamp) and HIV *gag* mRNA (Trizol) by qPCR and qRT-PCR (BioRad iTAQ). Two independent regions of *gag* were amplified and normalized to GAPDH genomic DNA or cDNA from the same sample (Figure 10AB, GAPDH data not shown). Intriguingly, some subclones lacking HIV *nef* transcripts still harbored gag proviral DNA, while in other subclones the HIV provirus was undetectable. Possible genomic DNA contamination was ruled out using controls lacking reverse transcriptase (no RT, Figure 10C). The segregation of positive and negative clones is clearly visible in the corresponding FISH contour plots for each subclone (Figure 10D). This is the first molecular evidence that RNA FISH:FLOW analyses accurately predict the HIV status of individual infected cells. Together the data indicate that both proviral genome silencing and genome deletion are occurring in 8E5 cells maintained in culture. Interestingly, the LAV provirus in 8E5 is integrated at

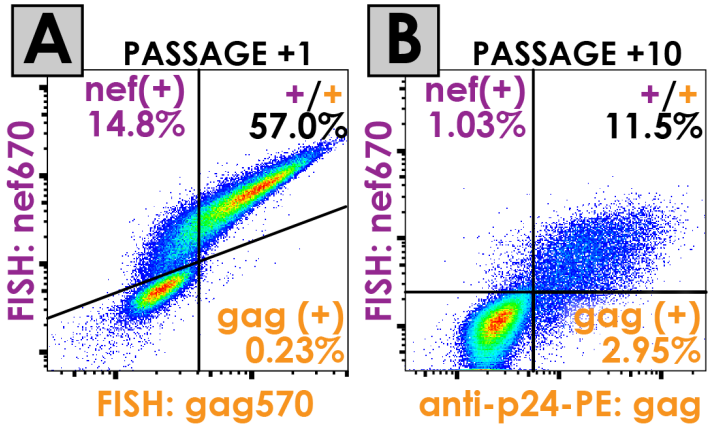


Figure 11. 8E5 cells commercially available demonstrate HIV heterogeneity and loss of penetrance over time. A Fresh 8E5 cells obtained from the AIDS Reagent Program were cultured for 5 days prior to analysis by FISH:FLOW with nef and gag probes. High dilution subculture for 10 passages (B) leads to loss of HIV transcription and gag protein production.

chromosome 13q14-q21[91], a site containing common fragile sites that would render this clone susceptible to proviral loss by genomic instability.

A fresh aliquot of 8E5 cells was obtained from the HIV AIDS Reagent Program to determine whether population heterogeneity might be a consistent feature of these cells. These 8E5 cells were tested by *nef* and *gag* RNA FISH within 5 days of their establishment in culture. The *nef*-positive gate constituted the main population of cells (72%, Figure 11A). Importantly, no cells stained positive for *gag* RNA without also expressing *nef* RNA in this multiplex assay as would be expected based on the staged transcription of HIV[28]. This representation highlights the ability of FISH:FLOW to discern among different stages of HIV infection (*gag* heterogeneity in the *nef*+ population). Subsequent passaging of the newly obtained cells at a 1:10 ratio led, by passage 10, to the reduction of *nef* and *gag* RNA-positive cells below 50% (not shown). Aggressive subculture by splitting the cells very low (bottleneck founder effect) accelerated the loss of *nef* transcription, where only 15% of 8E5 cells fresh from a public repository transcribed HIV *nef* by passage 10 (Figure 11B); importantly almost all of the *nef*-positive cells also stained p24-positive (Beckman anti-p24 KC57). These data demonstrate that FISH:FLOW is a surrogate assay for standard measures of HIV production and that the HIV transcriptional loss documented in 8E5 subclones is a reproducible characteristic of this cell line. I believe this observation reflects the strong selection of founder subclones that achieve spontaneous loss of HIV proviral DNA or silencing of transcription.

Model for loss of HIV penetrance in continuous 8E5 cultures

Together these data support a model where 8E5 cells acquire a selective advantage in continuous cell culture if they extinguish HIV expression, either by transcriptional silencing or by proviral genome loss. We suspect that stressing the cells during culture through delayed passage is likely to exacerbate this behavior, hastening a bottleneck of HIV-positive cells in the population. The loss of HIV from 8E5 cultures is of practical significance considering the widespread utilization of these cells for quantitation of HIV genome abundance in patient samples. My findings appear consistent with cautions raised in a recent analysis, which found variable numbers of proviral insertions within common latently-infected cell lines previously assumed to be homogeneous[92]. Furthermore, my specific findings on 8E5 cells were confirmed by a subsequent report using digital PCR[93]. My conclusions highlight the robust data these cell lines yield and underscore their intrinsic value to the field, but also support recent initiatives to validate common reagents in pursuit of reproducibility. The data show the FISH:FLOW method is a convenient means to rigorously validate the penetrance of HIV in the 8E5 starting population in any laboratory with access to a flow cytometer; without such validation, we suggest that quantification of HIV genomes using 8E5 cells should be restricted to relative comparisons only.

DISCUSSION

Worldwide, investigators studying persistence and therapeutic reactivation of latent HIV reservoirs are heavily invested in PCR readouts of genome quantitation. The FISH:FLOW method we have developed is one example of the very few tools

that allow quantification of HIV infection at the level of the individual cell; importantly this resolution is lost in PCR studies of cell-associated DNA or RNA from bulk populations. We feel strongly that a critical mass of laboratories investing in cell-level tools has not yet been reached, and the simple results presented here using a common cell line highlight the value of such studies as a complement to PCR approaches. Importantly, combining FISH:FLOW with antibody surface phenotyping meets the evermore urgent need to characterize specific cell subsets that harbor HIV in vivo. Multiple probe colors can assay different HIV transcripts in a single cell, allowing one to discern between early and late stage transcription, and increases the confidence that positive signal corresponds to intact provirus. The ability to then FACS-purify infected cells from human patient samples and study their transcriptional profile adds functional genomics to the growing list of possibilities. We feel these are opportunities not to be missed in context of the current challenges facing the field of HIV persistence and eradication.

CHAPTER THREE

Tractable viral outgrowth platforms to study host and viral adaptations during HIV1 cellular transmission

In Chapter 3, I describe viral outgrowth reporter cell lines to follow HIV replication in real time. This chapter has two sections:

Chapter 3A details construction and validation of TZM-gfp cells These experiments will be combined with some ongoing validation work on a sister cell line, TZM-GGR, and will be submitted for publication in the spring of 2018.

Chapter 3B is a combination of two short stories detailing the construction of lymphoid reporter cell lines for HIV infection. Clinical validation of the SupGGR cell platform is underway, with anticipated submission for publication in the summer of 2018. Dependent on the results of those experiments in a patient setting (HIV reservoir determination), the MoltGGR cell line will either be included in the same submission (novel lymphoid cellular tools as robust QVOA platforms), or will be submitted separately as a short report. Some additional work using outgrowth and titer of transmitted/founder viruses will be performed in both MoltGGR and SupGGR in the spring of 2018 to complete these manuscripts for submission.

The experiments described in this chapter are my own work, with important contributions from Shannon Caldwell, an electron microscopist in our laboratory. Shannon and I worked closely together to develop the correlative EM techniques described for targeted identification of HIV-infected foci.

CHAPTER 3A: TZM-gfp cells inform studies of HIV replication in vitro

INTRODUCTION

Emergence of HIV reservoirs

The past 3 decades of HIV research have yielded critical data that has saved countless lives, prevented millions of new infections, and brought the HIV pandemic under control in most developed nations. However, the speed of HIV research forced the virus 'underground' in human patients on ART, such that we eliminated bloodborne HIV before we understood anything about HIV replication in reservoir cells of the underlying tissues. These tissue reservoirs have now become the cells to beat, but we have little data on where to find them or how they harbor HIV long-term[39].

The tissue reservoirs established during acute HIV infection persist, and may evolve during the years and decades of ART therapy in a single patient[44]. These reservoir cells have become formidable adversaries in our fight to establish functional cures – that is, long term drug-free HIV remission. While it is recognized that a sterilizing cure – complete eradication of HIV from the body – is highly unlikely[94], targeted reduction of HIV burden in reservoir tissues will form the cornerstone of all efforts to reach a functional cure. However, we still lack the understanding of many such reservoir cells and similarly lack the specialized tools to

study HIV replication in these diverse cell populations. While memory T-cells form the bona fide latent reservoir (HIV provirus-positive cells that extinguish HIV transcription during the resting phase of cellular immunity), the 'expressed reservoir' such as that in myeloid subsets may remain transcriptionally active[39]. The pressures and mechanisms of transmission in these different cell types must obviously be different, and it is likely that HIV evolves means to excel in these distinct scenarios. The key difference that separates lymphoid from myeloid cells as potential reservoirs derives from the mechanism of latency establishment in lymphocytes.

Comparing characteristics of distinct cellular HIV reservoirs

Proliferating, activated lymphocytes become infected with HIV and generate new virions. The lifespan of an infected T-cell is estimated at ~48 hours[46]. Rare T-cells (including some HIV-positive) exit the cell cycle to form the memory lymphocyte subset, a process initiated by as yet unknown signals that results in shutting down NF-kB expression and consequent silencing of the HIV-1 LTR promoter[28]. Thus, in T-cells, HIV-1 expression requires host NF-kB expression, found only during activated proliferation of T-cells. Productive infection in lymphocytes is therefore a race against the clock, with the objective to generate offspring virions and infected daughter cells prior to G2 arrest and cell death induced by HIV-1 vpr[45]. The emergence of ART treatment has all but eliminated HIV viremia in treated patients: productively HIV-infected T-cells are depleted by attrition, while drugs prevent new infections by inactivating newly produced virions. Thus, the efficacy of

ART regimens depends on natural mechanisms by which HIV-infected cells die or are cleared by the immune system.

By contrast, myeloid cells such as macrophages are largely quiescent and lack the cell cycle machinery targeted by HIV vpr during infection[25,45]; these cells are not cleared by cytopathic effects of the virus, survive for months in culture[95], and would thus be spared in vivo by conventional attrition-dependent ART therapy. Infected macrophages also retain the capacity to transcribe HIV constitutively, and in the context of their longevity are hypothesized to form part of the expressed HIV reservoir during ART[39]. These characteristics make macrophages seemingly ideal candidates as reservoir cells in vivo, but the data supporting this hypothesis is fragmented and hotly contested. However, a growing number of investigators are becoming interested in the role of macrophages during HIV persistence and pathogenesis in vivo[96,97], especially in anatomic regions of relative immune privilege such as the brain[98].

Viral outgrowth assays as an estimate of in vivo HIV heterogeneity

In general, the studies to date of HIV outgrowth from blood-derived samples likely represent the 'low hanging fruit,' and rely on sampling pools of virus with a higher likelihood of replicating during proliferation-dependent in vitro culture assays such as the gold-standard QVOA (quantitative viral outgrowth assay)[78,99]. But other viral strains exist, including those comparatively unfit for replication ex vivo[99,100]. For example, the presence of HIV transcripts in AMs of the human airway was

suggested by our lab's early data[74], and has been repeatedly observed in vivo[74,95,96,101-112], but these findings were not immediately supported by straightforward outgrowth studies using standard cell lines at the point of care in Blantyre, Malawi. Such challenges in studying the outgrowth of more diverse HIV strains and reservoir cell types are reinforced by very recent data showing that humanized mouse models will detect and replicate certain HIV strains where industry standard culture assays fail[48,77,79]. Together the data suggest there is HIV present in vivo we are not equipped to capture by current outgrowth assays: either the infected cells or the virus itself may govern the fitness of these reservoirs to replicate in culture[99]. Given the non-renewable source of our patient material (BAL) and the need to iteratively optimize our outgrowth conditions to follow their progress in real time, my entry to the human subjects HIV persistence project in the laboratory of David G. Russell began as a study in reporter cell biology: can we see HIV infection and encourage its progress? You can't study virus replication if your assay says there isn't any.

Here I report the generation of multiple novel cell lines to follow the replication of HIV ex vivo using co-cultured primary macrophages as a source of virus. My overarching objective was to establish an outgrowth platform for use in studying BAL-derived HIV in AMs. In many cases I model anticipated scenarios, but recognize that some facets of human alveolar macrophage biology are not faithfully recapitulated by human monocyte-derived macrophages (HMDM). I apply these tools to established paradigms and open several lines of inquiry based

on the emergence of novel phenotypes.

RESULTS

Fluorescence readout for standardized HIV infectivity assays: TZM-gfp

JC53 cells are HeLa cell derivatives overexpressing the three major HIV co-receptors, CD4, CCR5 and CXCR4[81]. Subsequent work in the lab of John Kappes produced JC53-bl cells (later renamed TZM-bl), which added E.coli beta-galactosidase (β-gal) and firefly luciferase reporter genes to quantify inoculum infectivity[113]. The TZM-bl cell line has become a household reagent in HIV basic research, and has recently found expanded utility in the clinic as a sensitive viral outgrowth assay platform[114].

We previously pursued outgrowth of HIV from human BAL (BAL) in TZM-bl, CEM, and THP-1 cells, but found the experiments challenging to repeat and optimize, since these studies were performed in a clinical setting in Blantyre, Malawi, and used non-renewable human samples as input. The endpoint-nature of the qPCR, luciferase or β-gal assay (in TZM-bl) meant that outgrowth could not be followed in real time. Importantly, it remained unclear whether HIV in the human airway would exhibit macrophage- or lymphocyte-tropic character, or even whether human AMs would readily transfer infectious HIV to TZM-bl cells under these culture conditions. I reasoned that a visual readout in addition to quantitative reporters would enable the refinement of culture conditions to maximize the efficiency of HIV capture and longitudinal analyses even from limited input material.

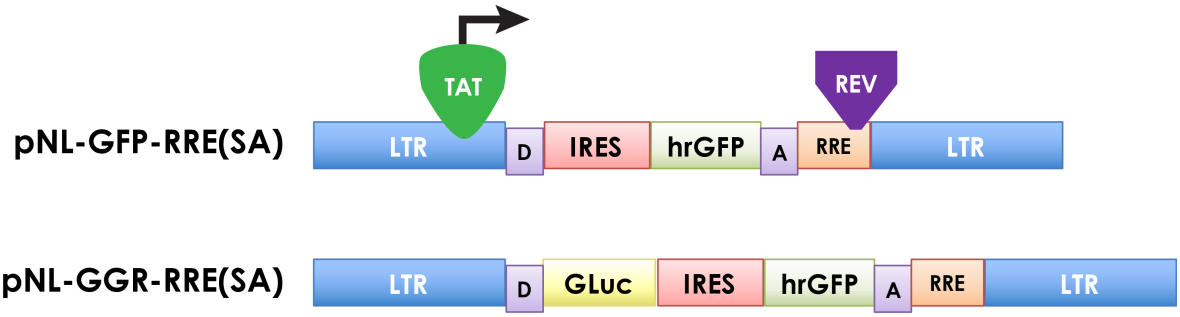


Figure 12. Lentiviral constructs dependent on HIV Tat and Rev expression for reporter signal. Top Fluorescence-only reporter expresses hrGFP downstream of the IRES. **Bottom** Bicistronic reporter expresses gaussia luciferase (GLuc) and hrGFP from a single messenger RNA. In the absence of HIV infection leaky transcription from the reporter proviral LTR produces messenger RNA. In these uninfected cells lacking Rev protein, cellular splicing machinery excises leaky reporter transcripts between the splicing donor (D) and acceptor (A) sequences. Conversely, productive infection with HIV generates Tat and Rev protein, which drive robust reporter transcription (Tat, green) and rapid nuclear export of RNA through the action of Rev (purple) binding the Rev response element (RRE, orange) on the nascent message. These exported transcripts are then translated and the reporter signal produced.

To this end, I re-visited the sample pipeline in Malawi where outgrowth cultures could be followed by fluorescence microscopy and flow cytometry, of which the latter is a notable strength among our research collaborators there[74]. Using parental JC53 cells (a kind gift of Dr. David Kabat, Oregon Health Sciences University) I engineered TZM-gfp cells using an HIV Tat- and Rev-responsive reporter virus pNL-GFP-RRE(SA)[115] (Fig. 12). The vector is a relatively recent and valuable addition to a large set of available transcriptional reporters of HIV output. However, transcription from the HIV-1 LTR can be leaky, generating background reporter transcripts in the absence of HIV infection. Notably, most current cellular reagents in widespread use (including TZM-bl, CEM-GFP and others) are exclusively LTR-driven, and thus subject to this background activation[116]. The pNL-GFP-RRE(SA)

vector was developed to include HIV-1 transcriptional splice sites flanking the reporter gene hrGFP, meaning both HIV-1 Tat and Rev proteins are required for fluorescence detection. In the absence of true HIV infection and Rev protein production, the hrGFP reporter gene is excised from the nascent transcript in the nucleus prior to export and ribosomal translation. In HIV-infected reporter cells the virus produces Tat, a transcriptional activator that drives transcription and accumulation of Rev protein (Fig. 12); Rev then binds the reporter proviral transcript and actively exports it, a splicing machinery "short circuit" that delivers full-length reporter gene message for translation and expression. The structure and fidelity of the pNL-GFP-RRE(SA) vector makes it an ideal choice for ex vivo HIV reporter assays.

Construction of TZM-gfp

Parental JC53 cells (a HeLa clone expressing CD4, CCR5, and CXCR4) were transduced with VSV/G-pseudotyped pNL-GFP-RRE(SA) reporter virions to derive a population of TZM-gfp cells in culture. Cells were then seeded at clonal density (0.5-0.7 cells per well in 96-well format), and cultured for further analyses. Wells with single foci were scored under low magnification by inverted phase contrast microscopy, and after 14 days were reseeded into replica 96-well plates. Validation of reporter activity was performed by infection for 4 days with HIV-1 ADA virus (a gift of Dr. Mario Stevenson, Miami CFAR), which is a CCR5- and macrophage-tropic HXB2 chimeric molecular clone isolated from the blood of an HIV-positive patient[117,118]. TZM-gfp clones were screened for strength of reporter output by fluorescence microscopy (Fig. 13). At this 4 day timepoint, widespread cytopathic effects were observed in most cultures (Fig. 13), including formation of multinucleate syncytia and many

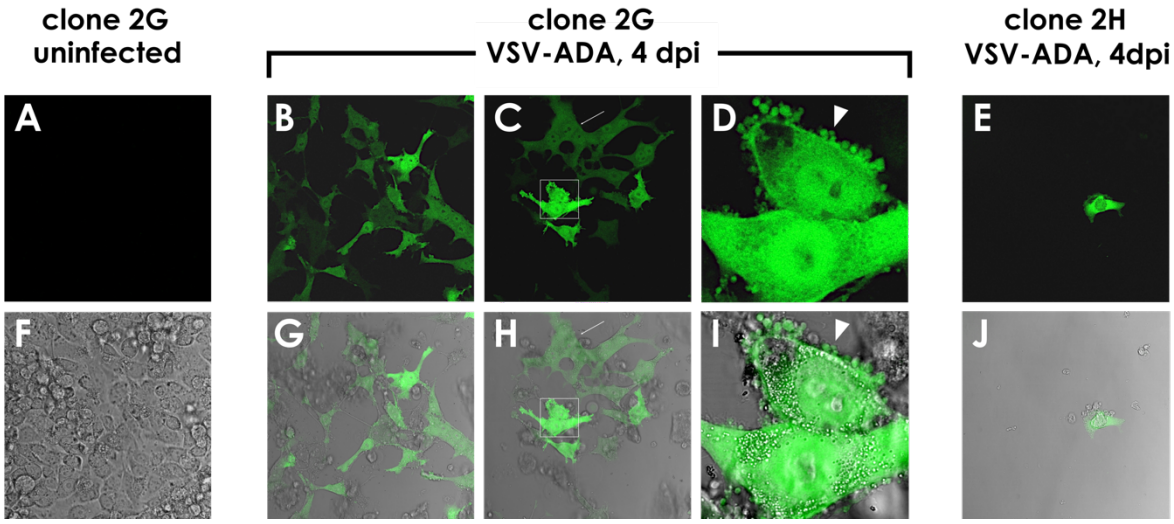


Figure 13. TZM-gfp clones are susceptible to HIV-1 infection and produce no background signal. A-D and **F-G** TZM-gfp clone 2G and E,J TZM-gfp clone 2H. A,F representative uninfected clone (2G) showing no background fluorescence signal in the absence of HIV infection. Infection with HIV induces strong reporter signal, multi-nucleate syncytia (arrow, C,H), and widespread cytopathic effects (note membrane blebbing, arrowhead, D,I). Infection in clone 2H occurs faster, much of the culture is detached or dead by 4 days post-infection (E,J).

detached free-floating cells. The two clones shown in Figure 13 (2G and 2H) were chosen for further analyses.

TZM-gfp cells report HIV infection of primary human macrophages

To establish the utility of TZM-gfp in reporting HIV infection from primary human macrophages, trypsin-harvested TZM-gfp reporter cells were added onto established monolayers of HMDM infected 14 days prior with VSV/G-ADA. Forty-eight hours after co-culture, cells were harvested and GFP-positivity was quantified by flow cytometry (BioRad S3e). At this timepoint, a threefold enrichment was observed in HIV (GFP) penetrance in clone 2H vs. 2G (29.8% vs. 10.3%), while the geometric mean signal to noise ratio was also greater in clone 2H (9.8-fold vs. 6.7-fold, respectively) (Fig. 14). On the basis of these results, clone 2H was designated

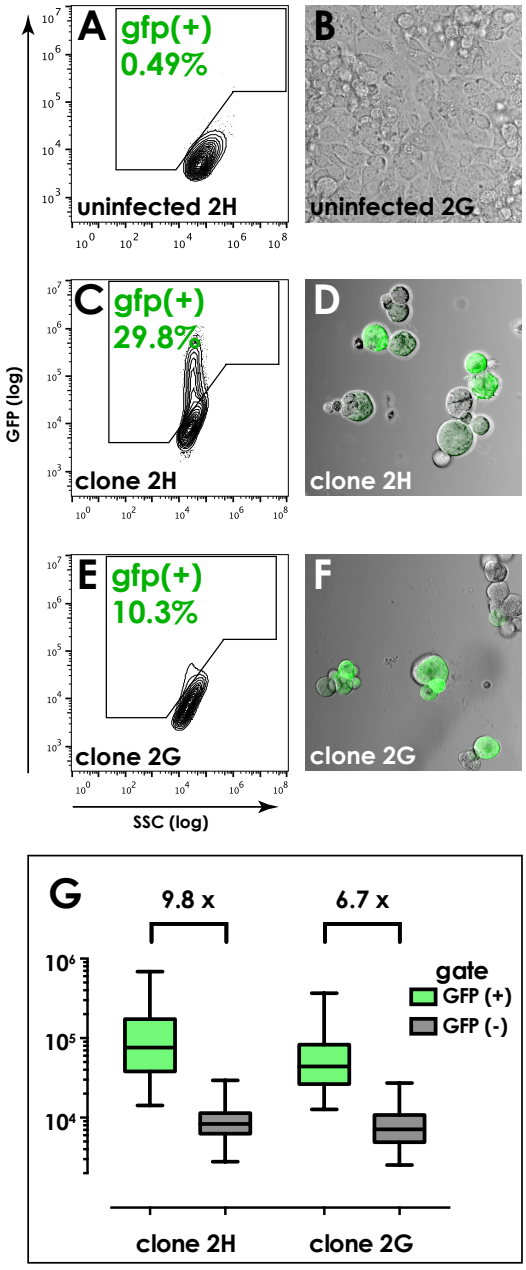


Figure 14. TZM-gfp cell clones report HIV transfer from infected HMDM at 48hours post-infection. **A,B** Uninfected TZM-gfp clones 2H and 2G reveal an absence of background signal or cytopathic effects. **C-F** Clone 2H exhibits higher HIV susceptibility on day 2 with greater induction and lower background, quantified in **G**.

'TZM-gfp' and used in all future analyses.

If TZM-gfp cells were to be used to report infectious HIV from human clinical samples in Malawi, the specificity of the signal was important – especially if infected cells are rare in human BAL. One would want to be sure a positive signal was real, and establish zero background in the absence of a true HIV infection. To address this, I co-cultured TZM-gfp cells and HMDM infected 7 days prior with various laboratory strains of HIV (Fig. 15). Widespread cytopathic effects in HMDM were observed 7 days post-infection with high titer VSV/G pseudotyped HIV-1 ADA stocks (MOI 20, 150 ng/mL p24, Fig. 15E-F), and to a much lesser extent with an inoculum of tenfold lower MOI (MOI 2, 12 ng/mL Fig. 15C-D). Interestingly this phenotype was not dependent on replication competent virus, since VSV/G-pseudotyped R9-Δ*env* also caused cytopathic effects at high titer in HMDM (MOI 20, Fig. 15G-H); VSV-R9-Δ*env* virions perform cell entry, integrate and transcribe, but lack an encoded envelope and thus do not package new virions in HMDM cells. Importantly, compared to robust development of GFP reporter signal in ADA-infected HMDM::TZM-gfp co-cultures, similar wells with R9-Δ*env* infection

developed no GFP fluorescence (Fig. 15G,H). However, the VSV/G-R9-Δ*env* virus can drive robust reporter expression if used for direct VSV/G-mediated infection of TZM-gfp (Fig. 15H, inset panel), showing the lack of signal in Fig. 15H is due to failed virus transfer from HMDM to TZM-gfp in the absence of an encoded envelope. These data highlight the strict regulation of the GFP reporter construct, and show that

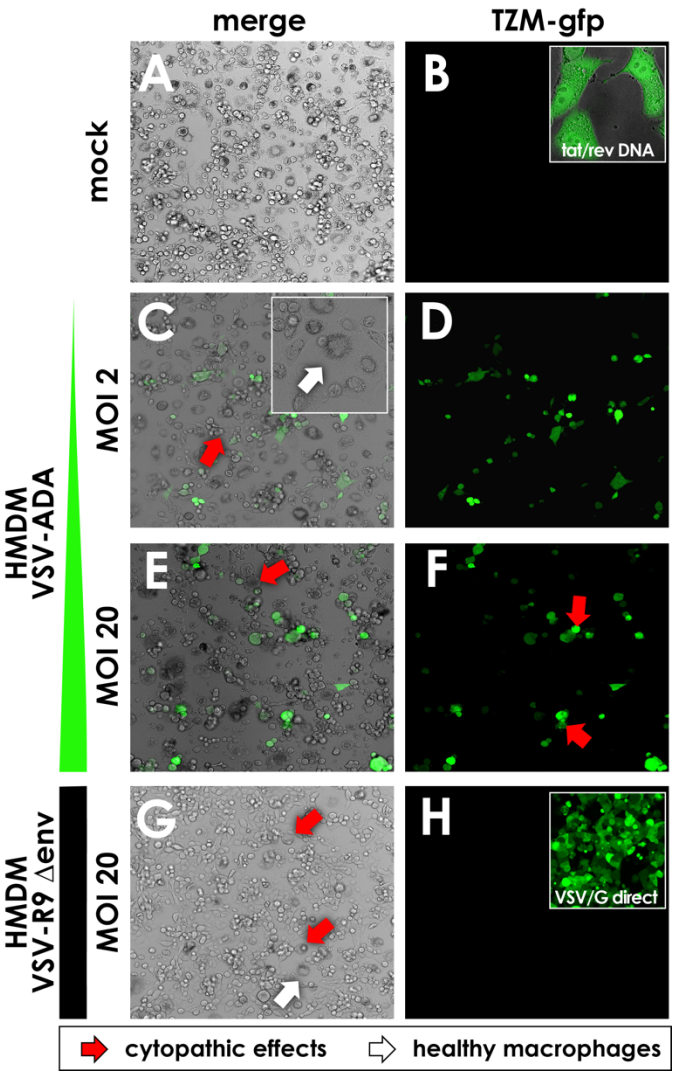


Figure 15. TZM-gfp reporter signal requires replicating HIV infection. HMDM were either mock-infected (**A-B**) or established for 5 days with two doses of VSV/G-ADA (**C-F**) or VSV/G-R9Δenv stock (**G,H**). TZM-gfp cells were then added for 72 hours prior to confocal imaging. **C-F** Replicating infection (ADA) induces dose-dependent cytopathic effects (red arrows, **C,E**) and GFP fluorescence in reporter TZM-gfp (**D,F**). Healthy macrophages are marked with white arrows. **G,H** VSV/G-R9Δenv pseudovirus is capable of entry and integration in HMDM but not forward replication (env deficient) in TZM-gfp and cannot drive development of GFP reporter signal (**H**) in cell to cell transfer. The VSV-R9 pseudovirus stock is single round infectious, however, as is causes macrophage cytopathic effects (**G**) and drives GFP reporter signal after direct VSV/G-mediated infection of TZM-gfp (**inset H**). HIV-uninfected cells that are transfected with tat+rev plasmid DNA express GFP reporter (**inset B**).

soluble Tat and/or Rev protein in the medium shed from cells harboring defective virus are insufficient to drive non-specific reporter expression in co-cultured TZM-gfp cells. By contrast, I show that Tat and Rev protein are sufficient to drive reporter GFP expression (Figure 15H, inset) by transfection of tat/rev plasmid DNA in HIV-uninfected TZM-gfp cells.

TZM-gfp assay refinement for durable reporter signal

While TZM-gfp cells added to cultured primary HMDM developed robust GFP reporter signal (Fig. 15), cytopathic effects and overall poor adhesion to the culture surface precluded longer-term culture for HIV harvest. Considering the dominant adherence properties of HMDM in culture, I hypothesized that incoming TZM-gfp cells in macrophage co-culture could not compete efficiently for the culture surface. To improve outgrowth conditions, I reversed the seeding strategy to overlay HIV-infected HMDM on top of an established TZM-gfp monolayer (Fig. 16). Different dilutions of TZM-gfp ($5 \times 10^3 – 1 \times 10^5$ cells/cm^2) were seeded in 35-mm imaging dishes (ibidi) in the morning and allowed to adhere. Four hours later, HMDMs infected 7 days prior with HIV-1 ADA in teflon screwtop jars (Savillex) were harvested by gentle pipetting, counted, and 100 cells were added to each 35mm TZM-gfp culture.

This reversed assay yielded a wealth of information about the infection behavior of macrophages and the performance of TZM-gfp cells in co-culture. First, it appeared that infection of TZM-gfp was primarily a local phenomenon, as the

vast majority of infection at 48h and 72h was focal (Figure 16B-E). Multinucleate syncytia formed by 48h, and continued to increase in size throughout the assay; by 120h there was evidence that cell-free virus had initiated infection independently of HMDM, triggering formation of multifocal syncytia that in many cases began to coalesce (Fig. 16F-K). This suggested that at the outset of the assay, virus was being efficiently transferred from HMDM to TZM-gfp by cell-to-cell transfer, with cell free virus infection proceeding with much slower kinetics. Indeed, associated with many primary syncytia at 48 hours was a single adhered HMDM (white arrows in Fig. 16B-E), presumably the offending HIV-infected cell of each focus. However, the GFP that filled the cytoplasm of cells within and surrounding the growing syncytium did not mix with the cytoplasm of the macrophage, indicating that HIV-infected HMDM initiate syncytium formation, but do not themselves form heterotypic fusions with TZM-gfp cells. The susceptibility of various types of cell membranes to HIV-mediated fusion has not been rigorously investigated, but this co-culture system seems ideally suited to such studies in the future. Given the importance of maximizing reporter cell sensitivity to capture trace HIV during reservoir studies in blood and tissue, the parameter of cell fusion and its relationship to HIV outgrowth seem fertile ground for follow up studies.

TZM-gfp infection is cell-density dependent

Interestingly, it was also clear that infection of TZM-gfp (as judged by reporter activity) was dependent on their seeding density. At lower seeding densities (50,000 and 100,000 cells per dish), robust GFP signal developed at multiple foci across the

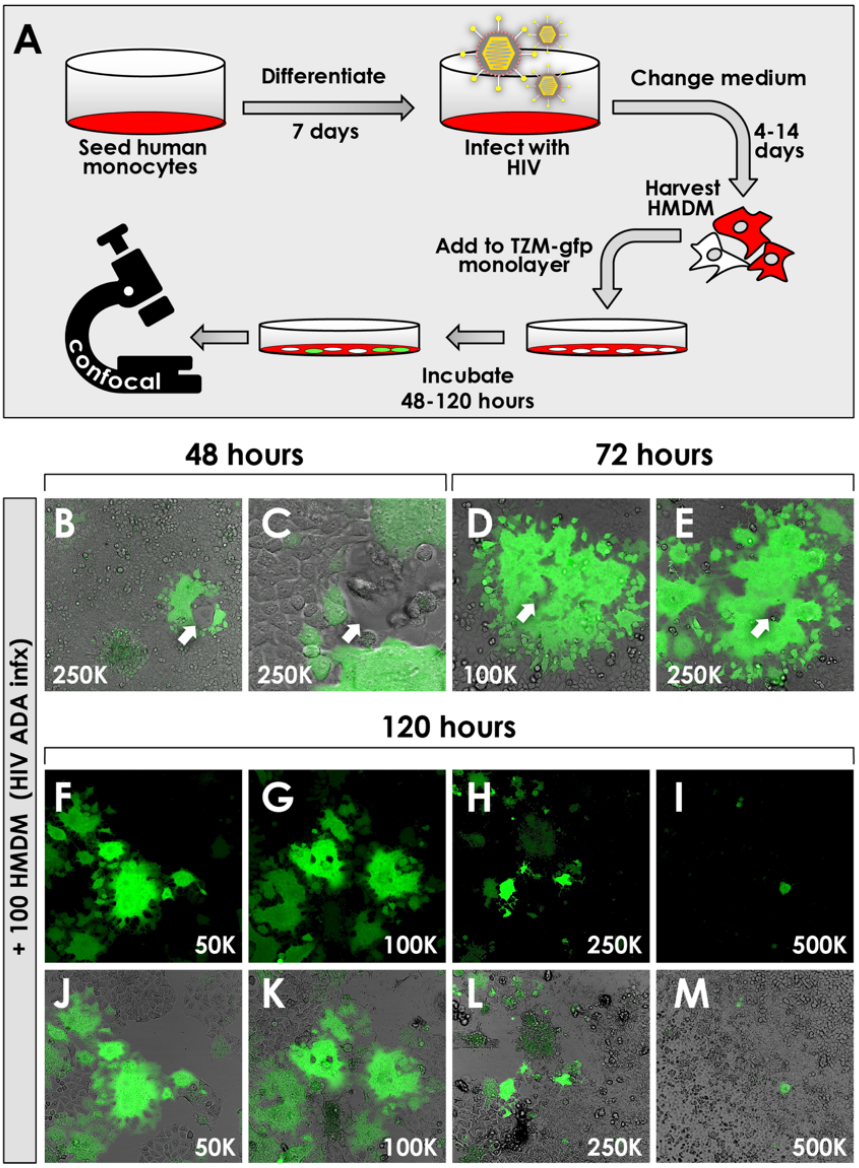


Figure 16. Focal infection of TZM-gfp by HMDM via cell-to-cell transfer is inhibited at high cell density. A Experimental setup showing infection of HMDM in Teflon jars for convenient harvest 4-14 days post-infection. Harvested HMDM are inoculated onto monolayers of TZM-gfp cells seeded at four densities (**B-E**), showing inhibition at high densities (**I,M** and also **H,L**). Number of seeded TZM-gfp cells is indicated in each panel.

culture surface by 48 hours (Fig. 16); much less infection was observed at higher density (500,000 cells per dish). In these experiments, an equal dose of 100 HMDMs was added to all wells with TZM-gfp dilutions. This suggested high density might inhibit the susceptibility of TZM-gfp to HIV-1 infection by cell-to-cell transfer from macrophages.

The impact of TZM-gfp cell density in permissiveness for HIV infection might be related to several factors, including the available surface area of target cell membrane, density inhibition of cell cycle, localization or quantity of HIV co-receptors or biophysical properties of membranes in tight proximity versus adequately spaced target cells. However, physical interaction of HIV-infected macrophages with target T-lymphocytes during virological synapse is a key requirement for cell-to-cell infection[119]. Thus, an alternative possibility is that overdense TZM-gfp cultures might be defective as targets for cell-to-cell infection, but unchanged in terms of susceptibility to canonical cell-free virus infection. To address this, I tested whether cell seeding density was a factor during cell-free HIV infection of TZM-gfp cells. Serial twofold dilutions of TZM-gfp cells were seeded in 2µL drops arrayed on optical bottom 35mm culture dishes (ibidi) and allowed to adhere overnight (1.1×10^6 – 5.0×10^1 cells/cm^2, or 20,480 cells – 1 cell per 2µL drop). The following morning, arrayed foci were overlaid with wild type HIV-1 ADA viral supernatant such that all drop cultures in a single dish were exposed to equal concentrations of the viral inoculum. At 72h post-infection cultures were analyzed by fluorescence microscopy. With increasing cell density, a dose-dependent

decrease was observed in infection outcome at the center of each cell focus (Fig. 17). These results matched those obtained with density inhibition of HIV infection in HMDM:TZM-gfp co-cultures (Fig. 16), suggesting the inhibition was related to viral entry or post-entry and independent of HMDM:TZM-gfp physical interaction. Interestingly, cells at the perimeter of each focus that were exposed to empty

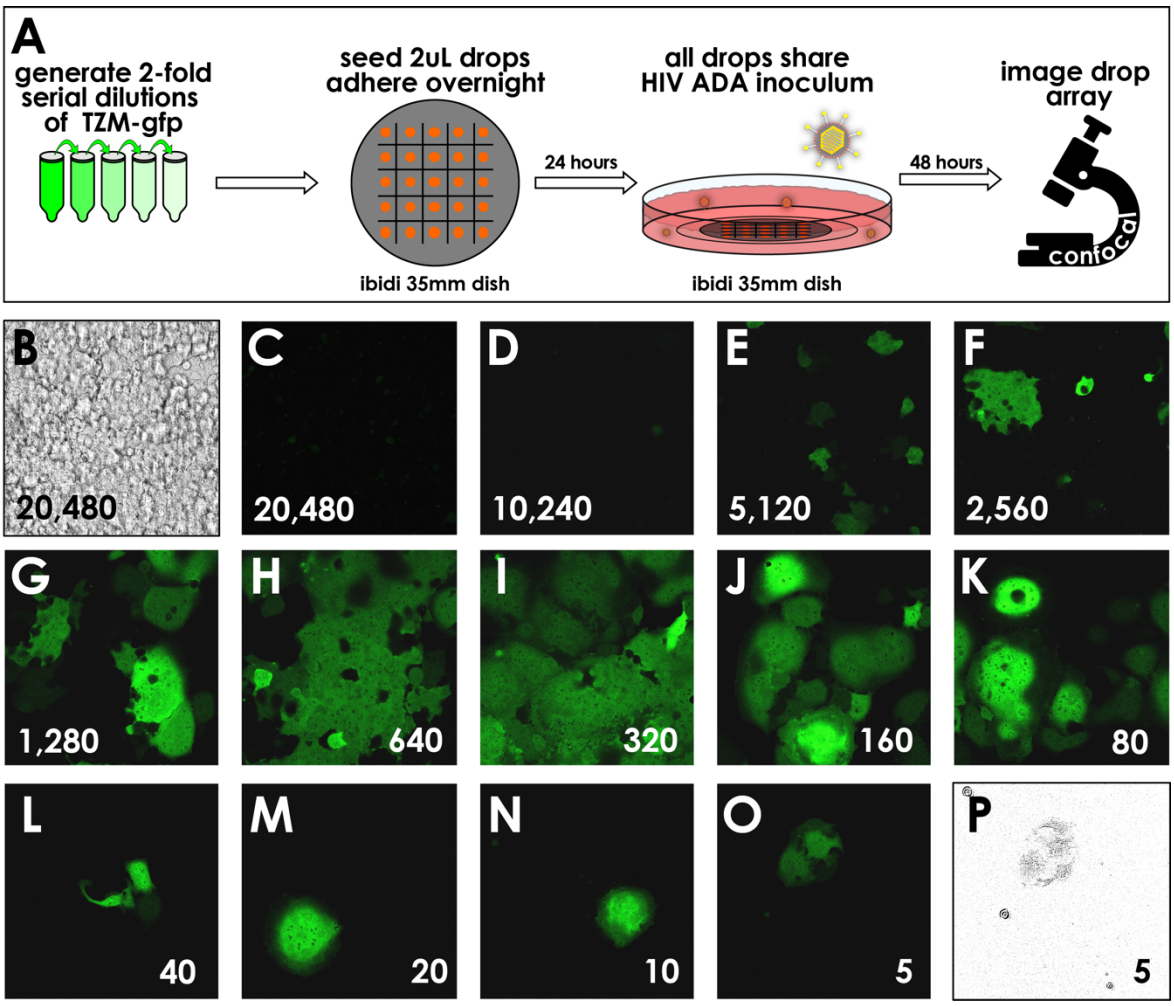


Figure 17. TZM-gfp cells are refractory to HIV infection at high cell density, and report HIV infection even at clonal density. A Serial dilutions of TZM-gfp were plated in 2uL drops on a 5 x 5 array in ibidi 35mm plates, and cells allowed to adhere overnight. Drop cultures were then overlaid with HIV-1 ADA supernatant such that all foci shared the same concentration of virus. Forty-eight hours post-infection, foci were examined for HIV infection. Brightfield images are shown for the highest (B) and lowers (P) dose of TZM-gfp cells. Signal in O represents HIV infection even when very few cells are plated. The number of cells plated per droplet are indicated in each panel.

culture surface on their apical edge became HIV-infected, independent of the central density of the focus (not shown), confirming that space constraints affected HIV-1 entry dynamics or the underlying transcriptional profile of spaced vs. packed TZM-gfp cells.

It was observed during earlier HMDM:TZM-gfp co-cultures that optimal infection outcomes were achieved at same day TZM-gfp seeding densities of 5 x 10^3 – 2 x 10^4 cells/cm^2 with absolute inhibition at 1 x 10^5 cells/cm^2 (Fig. 16). In drop culture assays of cell-free virus infection, the optimal range of seeding densities ranged from single cell density (~250 cells/cm^2) up to ~7.2 x 10^3, with absolute inhibition above 6 x 10^4 cells/cm^2 (Fig. 17D). Thus, despite significant technical differences, the effective density ranges of TZM-gfp HIV susceptibility returned by the two assays were comparable. Consistent with these findings, during the course of my studies, a report quantifying the optimal seeding density of the related TZM-bl cell line[120] suggested a linear range up to 3.1 x 10^4 cells/cm^2, with a precipitous loss of monolayer infection above 6.2 x 10^4 cells/cm^2. These results support my current findings and suggest an optimal seeding density between 5,000 and 20,000 cells/cm^2, with a preference toward lower densities if low numbers of incoming virions or HIV-infected co-culture cells are interrogated. This is highlighted by my later observations that cultures with very low numbers of HIV-positive foci will report HIV infection, but a large spreading infection throughout the well is precluded by the continued proliferation and eventual inhibition of uninfected reporter cells over several days of co-culture. Thus, a 'wait and see' approach for low-input inocula

may be a flawed strategy in the recovery of trace HIV from patient material, and upon positive foci identification, supernatants should be harvested early and used to inoculate fresh target cells at optimal densities.

This guidance overlooks the fact that HIV genetic variation may produce viruses with increased fitness for cell-free virus infection compared to other variants that rely on a higher efficiency transmission such as cell-to-cell infection[121]. A strategy to harvest viral supernatants from low-outcome primary co-cultures to passage onto lower density receiver cells might bias toward strains of high cell-free infection fitness, and may miss bona-fide HIV strains that replicate poorly in the absence of cell-to-cell transfer. Importantly, the well-established paradigm of cell-to-cell transfer from HIV-infected macrophages suggests that these cells may harbor strains with reduced pressure on cell-free infection kinetics, especially in macrophage-rich (and lymphocyte-poor) anatomical reservoirs such as the testes, lung, CNS and others. Studies to investigate human macrophages as potential HIV reservoirs should consider this potential handicap during assay design, and develop outgrowth strategies that emphasize cell-to-cell infection or risk underestimating the real HIV burden in tissue macrophages in vivo.

Analyses of single infection events with TZM-gfp

The TZM-gfp cell reagent is a scalable, versatile platform for the detection and outgrowth of HIV. However, the JC-53 parental cell line was developed prior to

our more advanced knowledge of the diverse HIV target cells in vivo or the key strains of HIV that predominate during chronic infection and person-to-person transmission of the virus, the so-called transmitted/founder (T/F) viruses[58]. The TZM platform is widely-employed and trusted for its reproducibility, but less is known about HIV genotypes that may be missed or underperform during outgrowth in TZM-related cell lines. In the context of low-input inocula such as might be obtained from human tissue macrophages, variable HIV fitness might prevent robust outgrowth if culture conditions are a poor surrogate for the in vivo environment to which the HIV strain in question is adapted. For this reason, it is said that all culture systems for quantitative viral outgrowth assays (QVOAs) can only serve as minimal estimates of the HIV reservoir or of the input inoculum[122]. However, since essentially no background GFP expression is observed in the absence of a true HIV infection, *any* reporter signal should be considered a potential HIV infection. This is true even if the virus produced is of insufficient fitness to replicate robustly in culture. In such a scenario, a valuable tool would be able to demonstrate virus production from rare infected cells despite the potentially poor replication capacity of the virus itself. I sought the means to capture HIV by cell-to-cell transfer, identify rare infected cells by reporter gene activity, and demonstrate the virus exists even if ex vivo culture conditions do not support robust replication.

Correlative electron microscopy of HIV-positive foci in TZM-gfp

To learn whether rare GFP-positive foci could be evaluated for productive HIV infection, I turned to correlative electron microscopy to image cells from such

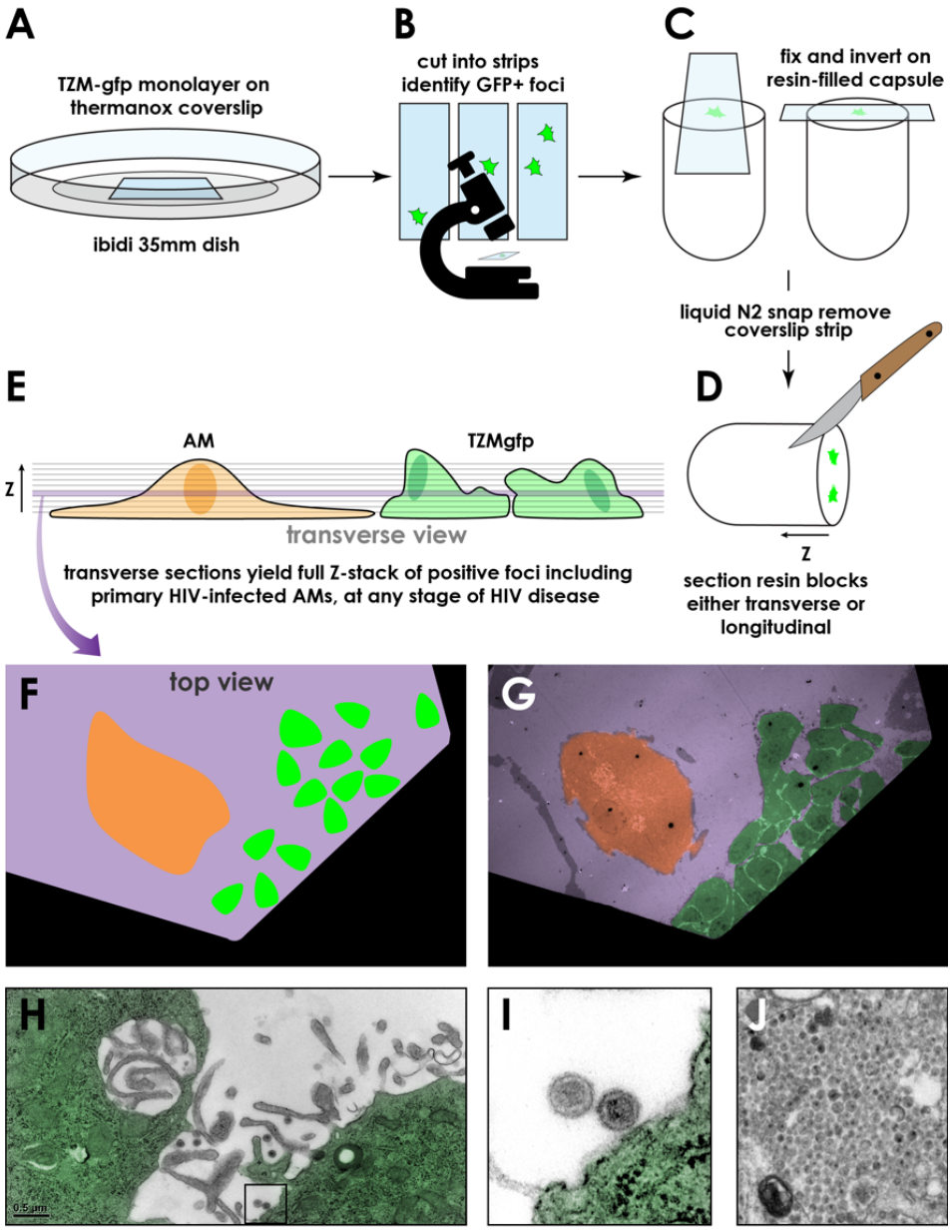


Figure 18. GFP-guided correlative electron microscopy identifies HIV virions in and around HMDM-associated foci. A-E Cultured TZM-gfp were co-cultured with 100 HIV-infected HMDM. Two days after plating, cultures were examined for reporter activity and regions chosen for embedding. Strips of coverslip were cut and embedded on top of a resin-filled Beem capsule, polymerized, then snapped off under liquid nitrogen. Sections were cut and EM analyses performed to view budding virions **H-I**. Previous analyses revealed the presence of VCCs within macrophage monoculture (**J**), but these structures were not observed in co-culture with TZM-gfp (**H-I**)

foci in HMDM:TZM-gfp co-cultures. HMDM were harvested from teflon jars as described above and 100 cells were seeded onto TZM-gfp monolayers on Thermanox coverslips (Nunc) cultured in 6-well dishes. A schematic of this experiment is shown in Fig. 18. At 72 hours after co-culture, GFP-positive foci were located by fluorescence microscopy; their location is readily marked with a hypodermic needle. Correlative microscopy coverslips with a pre-etched grid are also suitable for landmarking HIV-positive foci. Coverslips were fixed in glutaraldehyde and cut with scissors into 3-4mm strips, thinner than the width of a small Beem capsule. After EM staining and equilibrating in resin, each coverslip strip was inverted onto the rim of a filled Beem capsule such that liquid resin contacted the coverslip surface and extruded above the coverslip on either side between the capsule rim and the coverslip edge (Fig. 18). Polymerization of the resin was followed by snapping the plastic coverslip off the resin under liquid nitrogen, leaving embedded cells in the resin; this technique was developed in close collaboration with Shannon Caldwell in the Russell laboratory, to whom I am deeply grateful. Blocks were trimmed and sectioned from the apical side down, giving only a handful of sections per block, with potentially high yield data. Indeed, samples prepared and imaged in this way revealed HIV infection in TZM cells of identified loci (Fig. 18H,I), including assembly and budding of new HIV from the surface of TZM-gfp cells (Fig. 18I).

HIV within infected HMDM assembles and buds into intracellular virus-containing compartments (VCCs) continuous with the extracellular space and bounded by plasma membrane invaginations; the virus accumulates to large

numbers, visible as sacs of mature and maturing virions by transmission electron microscopy[123-126]. We have previously observed such VCCs in HMDM cultures infected with high-titer macrophage-tropic HIV supernatant (Fig. 18J). By contrast however, when co-cultured with TZM-gfp cells we found very few intracellular HIV virions within HMDM (not shown), despite the large numbers of extracellular and budding virions observed on and around TZM-gfp cells infected by HMDM. These results suggest that macrophage-resident VCC may be evacuated upon establishment of heterotypic cell contact, an interesting possibility that should be tested in future experiments. This scenario is consistent with existing observations showing that VCCs in macrophages align toward the point of T-cell contact in vitro and open during the synapse, ejecting a large number of accumulated HIV virions onto the lymphocyte surface at a time when it is most likely to become activated by antigen presentation[119,127-130]. This process requires actin cytoskeletal reorganization[131], and in macrophages can also be induced by exogenous ATP added to culture medium in the absence of any co-cultured cells[132]. Future work should investigate the efficiency of virion release from macrophages upon co-culture with various types of HIV-susceptible cells, and whether this process is at all influenced by viral genotype (see Chapter 5). For example, it is well established that HIV env directs the entry tropism of HIV for diverse target cell types, but less is known of how HIV adapts for efficient egress from specific cell types. There is evidence to suggest that viral fitness and adaptation in culture depends both on viral genotype but on host cell pressures as well[99]. For example, subcellular trafficking of env and matrix depends on sequences within gp41[133], mutations of which compromise

budding and accumulation of HIV into VCCs. Such lesions would impose a greater fitness cost on the virus in cells such as macrophages, where VCC-dependent cell-to-cell transfer predominates. Conversely, certain strains of virus may adapt toward cell-to-cell or synaptic transfer where the effective MOI on target cells is much higher thus reducing pressure on virion infectivity. The abundance of such viruses in vivo is unclear, but it is attractive to speculate that strains adapted to synaptic transfer would rely on this decreased burden for efficient replication, and hence may not be captured during in vitro outgrowth assays that rely on cell-free virus infection[122]. In vivo, such strains would still seed rebound viremia, but adaption for rapid outgrowth in different host cell types would take time, prolonging the period between ART interruption and emergence of rebound viremia. Indeed, these insights may offer a long-sought explanation for the lengthy period of drug-free remission in some of the more famous cases of HIV ART interruption. For example, the two "Boston patients", who lived for 12 and 32 weeks, respectively without ARV treatment or viral rebound, are unexplained exceptions to the relatively rapid (2-6 weeks) rebound in most patients following ART discontinuation. Indeed, investigators studying the rebound viremia of the Boston patients suggested that underlying macrophage infection may explain the duration of drug-free remission and the timing of eventual rebound in these patients[134].

DISCUSSION

The TZM-gfp platform was developed to provide the means to follow rare or low-frequency infection events during primary HIV outgrowth from potential

reservoir tissues. Intrinsic to this paradigm is the need to iteratively optimize the assay format for the HIV source being studied. A primary challenge here is the limiting nature of the human patient material, in our case BAL cells. The deployment of TZM-gfp to the point of care in Blantyre, Malawi (described below in Chapter 4) is a critical first step toward a robust assay for HIV infection in AMs of the human lung. My subsequent work with multiple cell lines, including adherent (HeLa- or HEK293-derived) and lymphoblastic cells (Molt4- and SupT1-derived) has revealed important features and caveats of each system. The predominant theme that emerged is the particular suitability of the TZM cell platform for macrophage co-culture assays. One reasonable hypothesis is that the solid-phase format of the assay (adherent macrophages and adherent reporter cells) maximizes the physical contact between the cells for efficient cell-to-cell transfer. I found that the seeding order (HMDM or TZM first) and the cell density in these co-culture assays determined the outcome and durability of the reporter signal, highlighting that the adherence properties of TZM-gfp cells are key considerations of the assay's performance.

Importantly, I show that TZM-gfp exhibit extremely low background signal with strong induction upon HIV infection. Both HIV Tat and Rev are required for this function, as these and other factors secreted from HIV Δenv-infected TZM-gfp cells are unable to drive reporter expression in uninfected bystander cells. This is an improvement over the standard TZM-bl cell line[113], which harbors the HIV LTR only and does not require Rev function.

The strict regulation of the GFP reporter in TZM-gfp cells makes identification of rare infection events possible. I applied this feature to develop a correlative electron microscopy workflow that would enable fluorescent location of rare HIV-infected foci in TZM-gfp:HMDM co-cultures, followed by targeted embedding and ultrastructural imaging of HIV virions in situ. This protocol allows investigators to derive proof of infectious HIV that may not meet the high burden of in vitro replication fitness shared among established viral outgrowth platforms. The contribution of such viral strains to HIV persistence remains unknown, but is a key paradigm as the field continues to categorize HIV genotypes associated with specific facets of transmission, persistence, or rebound. TZM-gfp and reporter cells like it are designed to capture these events, providing an opportunity to study viral reservoirs that might otherwise go undetected[48,77,79].

Future efforts with TZM-gfp cells in Blantyre, Malawi should focus on the quantification of potential HIV reservoir cells in the human airway. Proof-of-concept experiments are planned for the near future to infect cultured naïve human AMs with HIV ex vivo and test their performance in a TZM-gfp QVOA. Importantly, very recent work using TZM-bl cells[114] reported improved sensitivity in quantifying the HIV reservoir compared to standard QVOA assays. In support of these findings, the rapid kinetics and focal nature of HMDM-derived outgrowth in TZM-gfp cells shown here confirm the potential for this platform in clonal serial-dilution HIV outgrowth assays using primary human macrophages as input.

CHAPTER 3B:

Accessible quantitation of HIV reservoirs using improved cell lines with reporter activity

INTRODUCTION

Recent progress has been made toward streamlining quantitative viral outgrowth assays (QVOA)[78,114,135], which estimate the size of the HIV reservoir in the context of antiretroviral therapy (ART) or latency reversing agents (LRAs). The QVOA and other quantitative measures are key paradigms in developing strategies to reduce the HIV reservoir and thereby accelerate the path toward curative HIV therapy[39]. While several recently described assays exist to measure the size or changes in the HIV reservoir[48,85,122,136-138], the gold standard remains the QVOA. However, the standard QVOA protocol relies on human donor lymphoblasts and is therefore beset by donor variability, cost and scalability issues, while also relying on secondary readout such as p24 ELISA or qRT-PCR[78,135].

Recently, two separate reports describe improved cell line reagents to serve as surrogates for uninfected human lymphoblasts in QVOA pipelines[78,135]. These cell lines - SupT1/CCR5 (SupR5) and Molt4R5 (Molt4R5) – appear to offer enhanced stability and outgrowth properties compared to primary human lymphoblasts, are more convenient, and do not suffer the donor variability that can be observed in the classical QVOA platform. Despite these features, both reagents offer no direct quantitative readout of HIV replication, and still rely on nucleic acid amplification

or viral gag (p24) determination. Guided by the success of the TZM-gfp cell platform and the published, quantitative nature of the Tat/Rev responsive pNL-RRE(SA) vector series[115,116], I sought improvements to the SupR5 and Molt4R5 reagents that would enable reporter-based quantitation of HIV replication in QVOA-type assays.

RESULTS

Design concept of GGR-reporter lymphoid outgrowth platforms

To facilitate HIV reporter activity, I chose the reporter construct pNL-GGR-RRE(SA) (Fig. 12, hereinafter referred to as the 'GGR' construct) from the well-characterized Affinofile-GGR cell line, derived from HEK-293 cells[57]. The GGR construct expresses a secreted luciferase protein from the copepod *Gaussia princeps*, and the humanized recombinant green fluorescent protein from *Renilla reniformis* in a bicistronic IRES cassette. Importantly, gaussia luciferase (GLuc) is up to 1,000 times more sensitive than renilla or firefly luciferases, and is naturally secreted into the culture medium, enabling periodic sampling while sparing the reporter cells in culture[139,140]. Like other vectors of the pNL-RRE series, the reporter genes of the GGR construct are flanked by HIV splicing sites, meaning that any background transcripts downstream of the HIV-1 LTR in this provirus are spliced out in the absence of Rev protein.

SupR5 and M4R5 cells were each transduced with the GGR lentiviral vector (Fig. 12) and a panel of clones were generated by serial dilution. Individual clones were replica plated and selected for robust reporter gene expression (Fig. 19) after infection with HIV-nef-IRES-mCherry/BaL *env* (HIV-Cherry). Flow cytometric analyses at early timepoints (48 and 72 hours post-infection) demonstrated that many cells expressed mCherry while a subset of those were also GFP-positive, suggesting early infection (mCherry-positive only) could be distinguished from cells at later stages of infection (double positive). Importantly, in both cases very few cells expressed GFP in the absence of HIV-Cherry expression (Fig. 19) showing strong coordination of the GFP reporter gene with the virus driving its expression in these reporter cells.

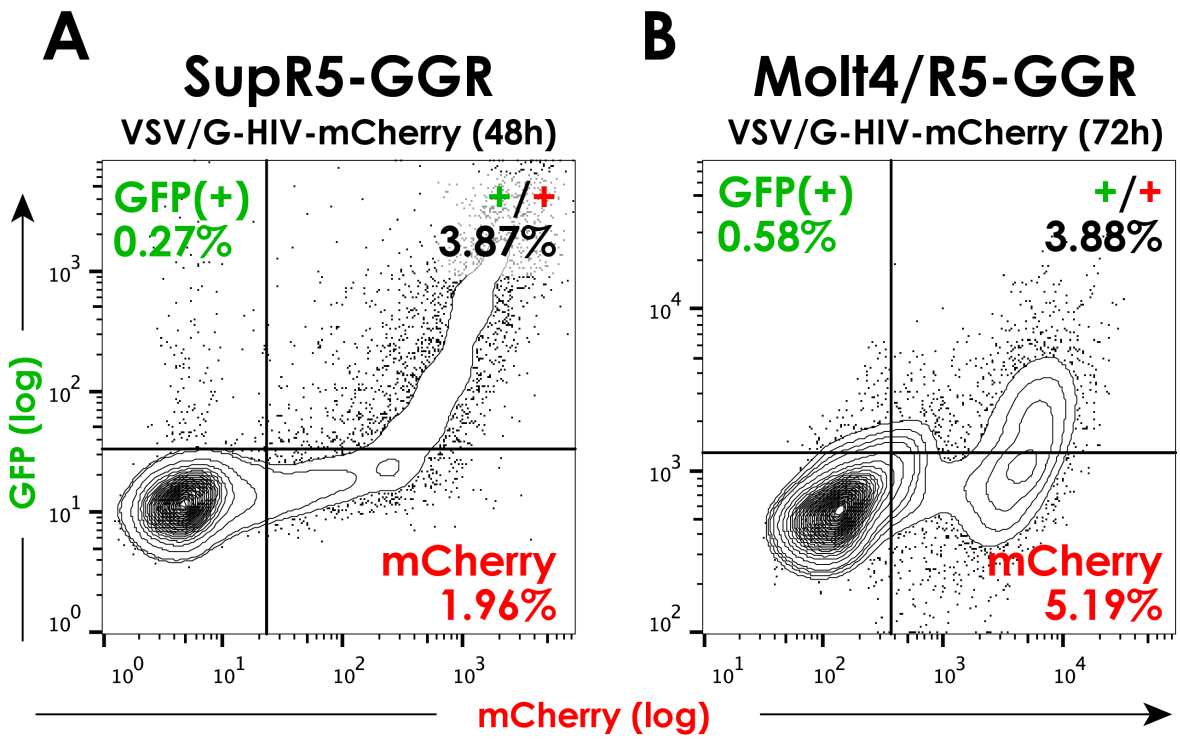


Figure 19. SupGGR and MoltGGR cells report HIV infection at early timepoints post-inoculation. A SupGGR cells were infected with VSV/G HIV-mCherry stocks for 48 hours prior to analysis by flow cytometry for GFP reporter signal and HIV-mCherry signal. **B** Similar analyses performed in MoltGGR cells infected for 72 hours with the same virus.

Comparative HIV susceptibility in MoltGGR and SupGGR

To compare the sensitivity of these two cell lines with the standard TZM-bl cell platform, a titration series of cell-free virus stock (wild type HIV-mCherry) was used to infect each cell line (standing infection without DEAE-Dextran or spinoculation). Seventy-two hours post-infection, HIV-positive wells were scored for each dilution and the TCID50 calculated for this stock in each cell line tested[141]. I found the TCID50 of the HIV-Cherry stock was comparable in SupGGR and MoltGGR cells (6.4 x 10^3 vs 5.0 x 10^3, respectively, Fig. 20), versus that in TZM-bl cells of 9.0 x 10^3. Infections of lymphoid cells are usually carried out using spinoculation and in the presence of a polycation (DEAE-dextran or polybrene) to enhance virus binding, which would

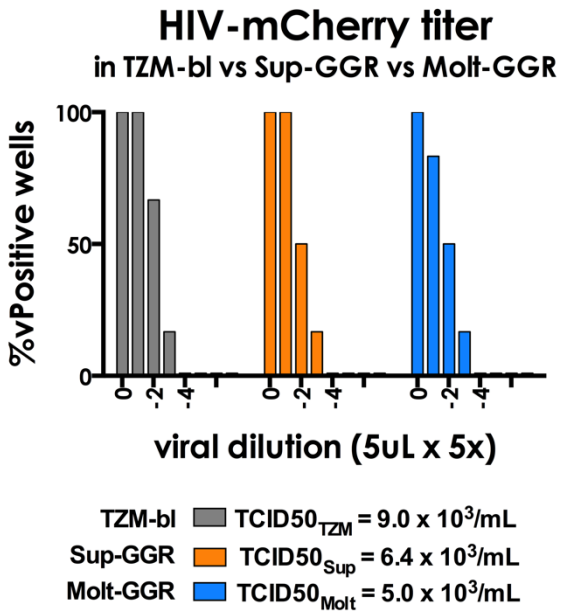


Figure 20. Titration of HIV-mCherry(BaL) in MoltGGR, SupGGR and TZM-bl cells. Fivefold dilutions of viral stock were added to cultures without additives or spinoculation to directly compare infectivity. Titer was calculated as TCID50 according to the Reed-Muench method).

significantly increase the performance of these cells in this titration assay. However, here these procedures were omitted to compare all cell lines tested using as unbiased approach as possible. Nevertheless, more rigorous testing of HIV susceptibility for these platforms with various strains of virus is presented below.

The Molt4-CCR5 cell line was reported by Dr. Bob Siliciano[78] as a surrogate for human peripheral blood lymphocytes in their quantitative viral outgrowth assays, and is now used exclusively in their laboratory for human patient assays. To validate the new MoltGGR cell reagent for use with human HIV isolates, I performed a stock titration experiment using 27ZP, a Clade C HIV strain I isolated from the peripheral blood of an HIV-positive human subject in Blantyre, Malawi. In a first experiment, I sought to demonstrate the quantitative nature of the fluorescent GFP readout. Fivefold serial dilutions of strain 27ZP were inoculated in replicate wells (96-well format) of cultured MoltGGR cells, and the cultures were incubated for 8 days. Wells were harvested by simple pipetting and analyzed for GFP fluorescence on a BioRad S3e instrument. GFP-positive cells were gated and the fraction positive was plotted as a function of dilution (Fig. 21). A log plot of the dilution series reveals a highly correlated ($R^2 = 0.9995$) linear region, suggesting the GFP readout provides robust quantitation of HIV infection.

The MoltGGR platform was designed to provide convenient means to capture HIV from co-cultured human tissue cells (notably macrophages) by viral outgrowth. Ideally a portion of the suspension MoltGGR cells could be readily

sampled to monitor outgrowth by flow cytometry, without sacrificing the co-cultured primary human cells in the same well. The strong linear correlation returned by the limiting dilution series with low numbers of sampled MoltGGR cells (96-well format) is an important proof of concept using a primary human HIV isolate (27ZP).

To expand the characterization of MoltGGR cells, the gaussia luciferase (Gluc) assay was tested with a dilution series of the same 27ZP viral stock. Serial dilutions were inoculated in MoltGGR cells (96-well format) and incubated for 8 days without medium changes. Supernatant was harvested from the top of each well at three and eight days post-infection for GLuc assay, without disturbing the cultured cells beneath. A particular feature of the GGR platform is the capacity for longitudinal sampling, which enables high-throughput screening for virus-positive wells prior to harvest for downstream analyses. This feature enables the early identification of outgrowth for subsequent molecular confirmation by *env* cloning or single genome amplification (SGA); importantly, a key focus is the generation of early isolates that have not undergone cell culture adaptation or accumulated mutations. The sensitive Gluc readout enables such analyses, and can provide rapid, high throughput detection in minutes using this accessible assay. Figure 21C shows the results of GLuc reporter activity at two timepoints post-infection. The linear region of the titration plot reveals that higher concentrations of viral inoculum yield detectable results by day 3, but that some wells harboring HIV are not detectable until day 8 (compare dark vs. light blue curves of Fig. 21C). No additional wells became GLuc-positive after day 8, suggesting this cell-free virus titration assay was

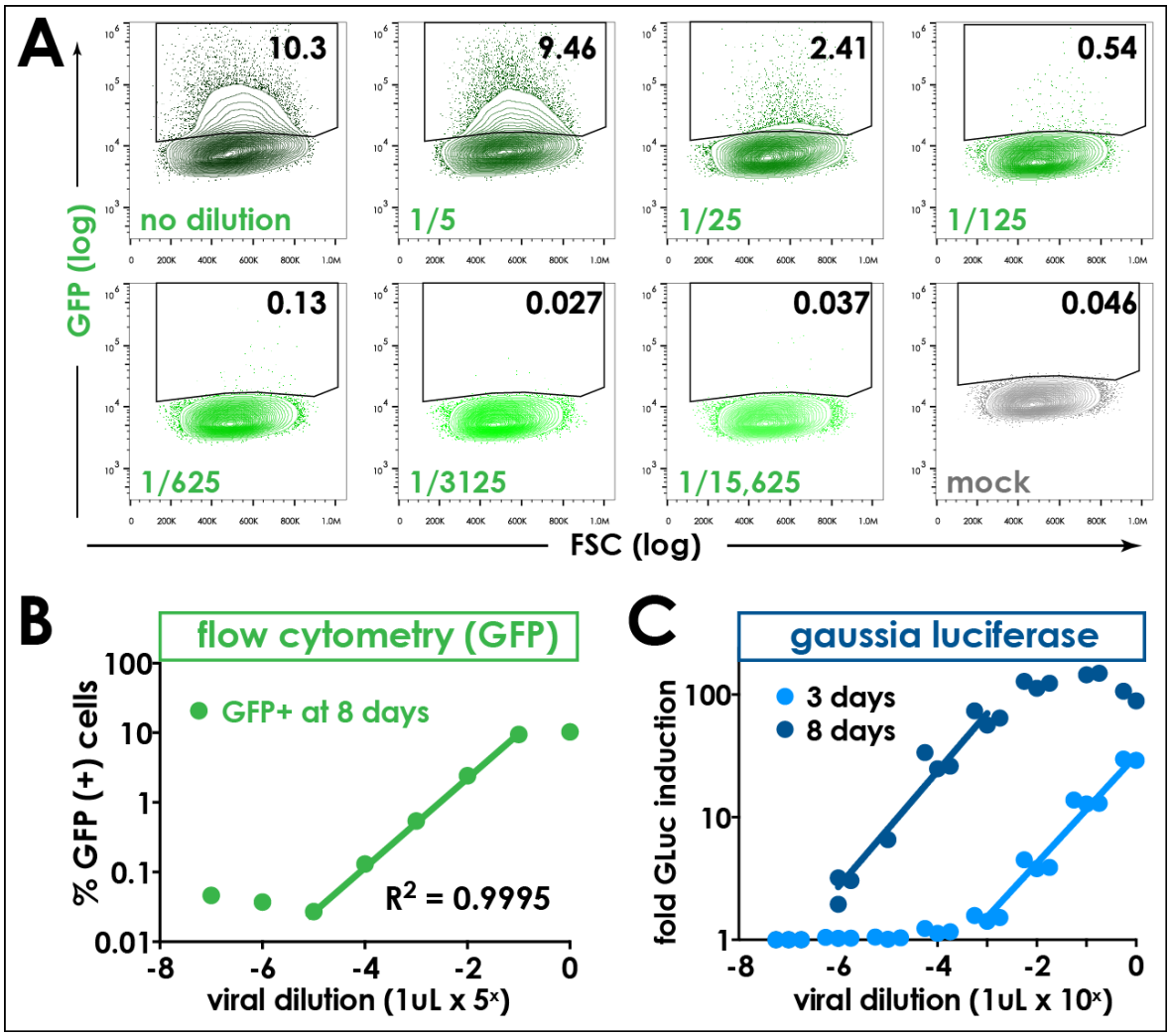


Figure 21. Quantitative detection of primary Clade C isolate 27ZP in MoltGGR cells. **A** Fivefold dilution series of cell free viral stock in MoltGGR cells cultured for 8 days post-infection. The GFP-positive fraction for each dilution is indicated in black (as a percentage), with the corresponding dilution indicated in green text. Before flow cytometry, cells were harvested from 96-well culture format with simple pipetting. **B** Plot of log percent GFP-positive fraction from **A** versus $\log_5$ viral dilution showing high correlation in the linear region of the curve. **C** A similar experiment using serial tenfold dilutions of 27ZP in MoltGGR cells. Supernatants were sampled in triplicate on day 3 and day 8 to quantify GLuc activity without disturbing the cultured cells. Titration curve shows saturation at three highest doses on day 8, with absence of signal at the lowest dose.

maximally sensitive by this point. However, further work is necessary to robustly discern the time to reliable detection using a daily GLuc timecourse of many single virion-inoculated wells of MoltGGR cells.

Single cell inoculum quantitative viral outgrowth assay in MoltGGR cells

The performance of the MoltGGR system as a viral outgrowth platform depends on a single HIV-infected cell initiating replication in an individual well. To model this scenario, I generated input cells by infecting TZM-gfp cells with human Clade C PBMC isolate 27ZP for 5 days. Cells were trypsinized and the proportion of GFP-positive cells was established by flow cytometry (~10.2%, Fig. 22A). Unsorted cells from the infected population were then diluted to a frequency of 0.75 cells per 50uL, such that about 75% of the wells on the plate were expected to receive a single cell, with about 10% of these being GFP-positive. Wells pre-seeded with $5x10^3$ MoltGGR cells were then inoculated with 50µL of cell suspension. For a 96-well plate plated at 75% density with a GFP penetrance of 10% one would expect 7-8 wells positive for HIV outgrowth (96 wells x 0.75 x 0.10 = 7.2). Under these conditions eight wells were detected by GLuc assay at 3 days post co-culture, using the Tukey 1.5x interquartile range (IQR) to establish significantly induced wells as 'outliers' (Fig. 23B). For this population, there was a very small IQR, confirming most wells (~90%) were uniformly negative for GLuc activity. This small IQR spread revealed a range of induction (2- to 36-fold induction above background, mean 14-fold) among the GLuc-positive wells on day three. This induction range at early timepoints (3 days) is likely driven by differences in HIV lifecycle within single TZM-gfp cells plated, where

input cells harboring late stage HIV infection express the GFP reporter and produce virions immediately upon co-culture. By contrast, very early infections prior to integration would exhibit a delay before production of new virus and by GFP estimate of HIV status would score false negative at the time of seeding, causing an underestimate of the true HIV-infected population by flow cytometry. The GGR induction range increased (4- to 130-fold above background, mean 37-fold) by day seven (Fig. 22B), with the emergence of additional GLuc- positive wells (total to 13/96 = 13.5% positive vs. 10.2% GFP scored on day of seeding). As with the cell-free titration experiment detailed above (Fig. 21C), it will be important to establish the timepoint at which the MoltGGR QVOA assay is maximally sensitive by daily longitudinal sampling using a wider panel of viruses (see Future Directions).

Analyzing GGR reporter induction on day 3, I compared outgrowth from single TZM-gfp cells infected with either 27ZP or HIV-1 BaL. Interestingly, among the wells scoring positive for BaL infection, GLuc induction on day 3 was lower by twofold on average than in positive wells harboring a single 27ZP-infected cell (Fig. 22B). This finding suggests genetic variation among different HIV strains may drive fitness for cell-to-cell transfer in this outgrowth assay, which may be a strong platform to identify and characterize the sequences responsible. The high efficiency of cell-to-cell transfer means that reporter signal at early timepoints derives predominately from this mode of infection during co-culture outgrowth assays. This is an important future application of the assay, since essentially nothing is known about cell transmission fitness at the level of the viral genotype.

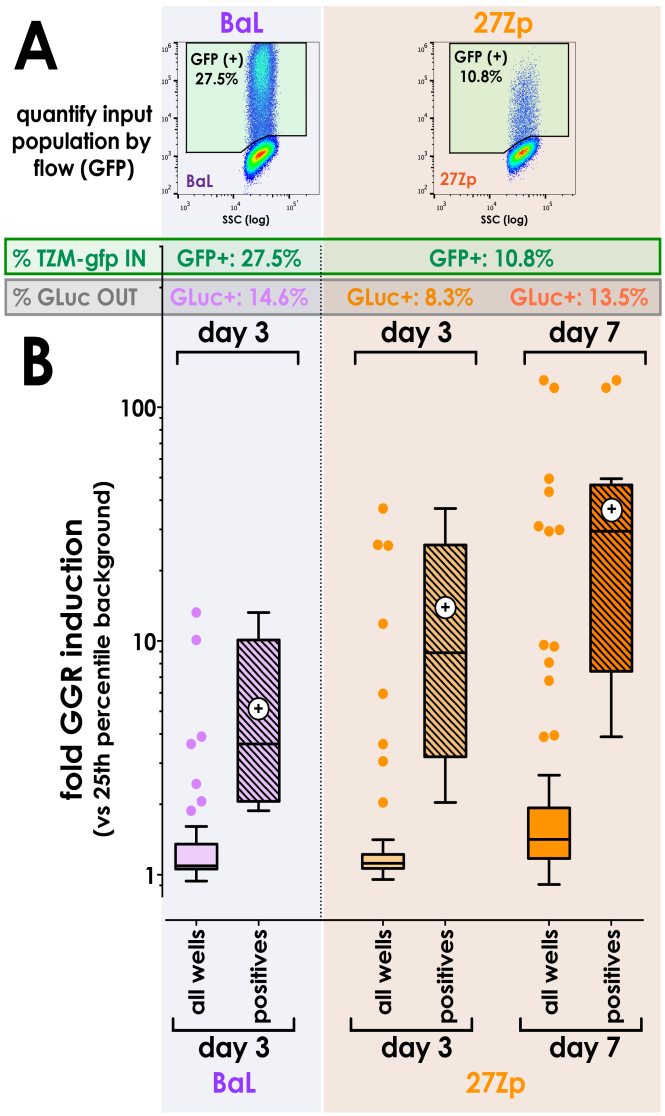


Figure 22. Single HIV infected cells seed a robust quantitative viral outgrowth assay in MoltGGR cells. **A** TZM-gfp cells were infected with the indicated virus stocks and GFP-positive cells were quantified as an estimate of true HIV penetrance. The bulk TZM-gfp population was then seeded at single cell density to quantify HIV outgrowth by MoltGGR QVOA. Input GFP penetrance is indicated in green, the resulting percentage GLuc-positive fraction for each timepoint indicated in the grey box below. **B** Tukey statistical analyses reveals outliers (> 1.5 IQR) that harbor GLuc activity. GLuc signal for the whole seeded population is plotted for each virus and timepoint (solid color bars), with GLuc-positive outliers marked above (colored dots). appearing above each box. Boxes represent the interquartile range (25-75 percentile), with median drawn as a straight line. Whiskers delineate the 1.5x IQR, and the geometric mean is indicated by the round '+' symbol. All wells scoring positive were then re-plotted to show their distribution (hatched bars). The distribution of positives at early timepoints reveals that single HIV-infected BaL cells transfer HIV and induce reporter signal twofold less efficiently than 27ZP cells, a key metric that highlights an interesting application of this assay to study cell-to-cell transmission of different viral genotypes.

Experiments are now underway to interrogate a panel of transmitted/founder (T/F) HIV clones for cell-to-cell transfer fitness using variations on this MoltGGR QVOA theme, and to determine the time to maximum sensitivity described above. The power of this type of assay combines quantitative population studies (how many cells are infected?) with qualitative features such as propensity for cell-to-cell transmission and drug/neutralizing antibody susceptibility.

Since the readout in this assay derives from HIV outgrowth seeded by a single cell, much resolution can be gained by assigning specific viral mutations to observed outgrowth phenotypes. For example, primary HIV isolates may replicate poorly in vitro, and may suffer extinction or adapt to culture conditions over time[142]. The sensitive single cell platform described here identifies viral clones early during their replication ex-vivo (low genetic diversity), and can be used to compare the outgrowth kinetics of these early isolates with progeny, culture-adapted virus. The existing longitudinal data pertaining to sequence variation that drives performance in cell culture, suggests that HIV adaptation is dependent on the cell host an though. However, this is an important question since all labs employing QVOA-type assays admit these platforms are at best a minimal estimate of the virus burden in vivo: many strains will fall below detection thresholds of standard outgrowth assays and be lost in subsequent analyses.

Refinement of the SupGGR platform

From the original heterogeneous SupGGR population, clones were seeded

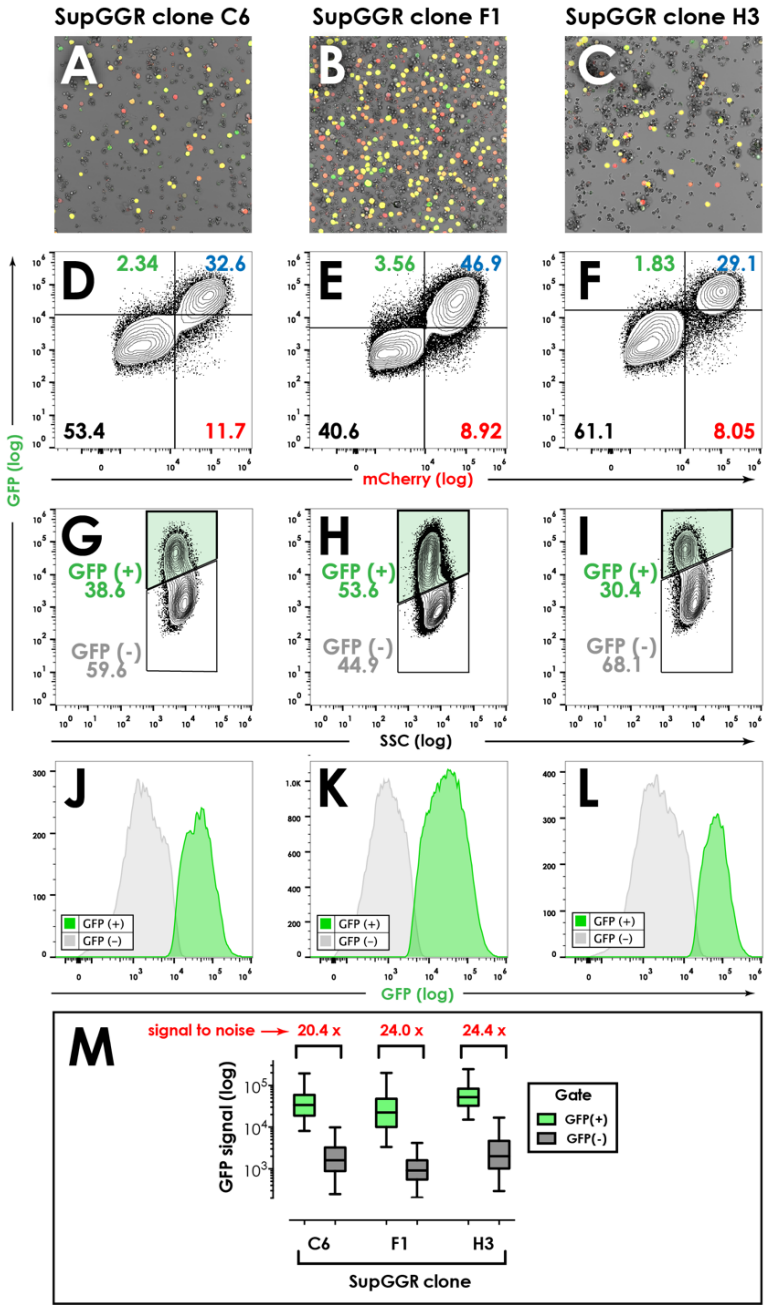


Figure 23. Characterization of SupGGR clone HIV susceptibility and reporter induction. A-C Confocal images of the indicated SupGGR clones infected with VSV HIV-mCherry. Clone F1 is more susceptible to HIV infection assessed by fluorescence microscopy (**B**), flow cytometry (**E**)and by florescence signal to noise quantitative analyses **H**,**K**,**M**. In **M**, positive and negative gates were analyzed for median and interquartile range fluorescence intensities, with the range of signal reflected by the whiskers in plot **M**. Clone F1 was chosen for further analyses based on high GFP induction, lowest and least spread background (GFP-negative gate, **grey bar** in **M**). Signal to noise ratios for each clone are indicated in **red** text.

and expanded for characterization. A panel of 55 clones was interrogated for reporter induction upon HIV infection, and three of the strongest inducers were chosen for more thorough analyses, clones H3, C6, and F1. These clones were spinoculated for 2 hours with stocks of VSV- HIV-mCherry, and cultured for six days post-infection. Cells were then imaged by confocal microscopy and in parallel analyzed by flow cytometry for reporter induction.

Imaging analyses revealed that a high fraction of SupGGR F1 cells became infected with HIV-mCherry, with predominantly co-localized GFP signal (Fig. 23). This data was confirmed by quantitative flow cytometric analyses, showing an increased penetrance of HIV infection by 48 hours (53.6% GFP penetrance, vs 38.6% and 30.4% for clones C6 and F1, respectively, Fig. 23A-I). As a caveat, VSV/G pseudotyped HIV-mCherry was used in this experiment to quantify reporter activation absent varied efficiency of HIV entry among the clones tested. However, SupGGR cells were later extensively tested with wild-type HIV stocks to confirm their utility in outgrowth assays. Clone F1 also exhibited the lowest background fluorescence (compare grey bars in Fig. 23M) with the least amount of spread around the median background. This property becomes important for assays of co-culture, where reporter cell background complicates gating strategies to separate primary cells from reporter cells. Clone F1 and H3 had higher signal to noise ratios than clone C6 (red text in Fig. 23M), despite the highest median fluorescence in the GFP-positive gate of clone C6. With these data, clone F1 was designated 'SupGGR' and advanced for further analyses.

Validation of SupGGR cell performance using relevant HIV strains

The MoltGGR platform performed very well in my hands for the outgrowth of both lab-adapted HIV and primary virus isolated from Malawian adults (27ZP). However, recent reports describing the SupT1/CCR5 as an alternative outgrowth platform pursued these cells after variable success with MoltGGR cells in primary outgrowth assays[135], a concern echoed by others in the field [D. Richman, UC San Diego, personal communication]. Building on these findings, I sought to expand the utility of the SupGGR cell line by characterizing the outgrowth of highly relevant, early isolates of HIV such as those encountered during reservoir quantification studies using patient-centered QVOA assays.

High content sequencing technology has made possible the sequence determination of many HIV strains within a single patient, the hierarchical clustering of which can illuminate the sequence of viruses that most likely initiated HIV infection in that patient. Such ancestral strains are the strongest candidates for the viruses that are actually transmitted from person to person, and defining their biology for large groups of patients can help us learn potential targets for prevention of transmission. These have been coined transmitted/founder viruses (T/F)[58], and have also been subsequently shown to comprise much of the early rebounding HIV burden upon therapy interruption[143,144]. Given the very limited knowledge we have of T/F virus biology, there remain many key unanswered questions about these special HIV strains, particularly their adaptations to different cell types and the subtleties of their transmission between cells. Studying these

strains using a tractable, biologically rich platform such as that described here will yield extremely valuable insight into potential future targets.

An expanded outgrowth toolkit: SupGFP and TZM-GGR

I generated two additional reagents, SupGFP and TZM-GGR reporter cells, using an identical workflow as that described above for SupGGR and TZM-gfp cells. SupGFP (clone 8C) cells harbor the pNL-GFP-RRE(SA) reporter vector and do not express gaussia luciferase or any enzymatic reporter. Conversely, TZM-GGR cells (clone 4H12) harbor the GGR construct expressing both gaussia luciferase and GFP. I do not describe the generation of these cells here in detail here, but validate their use below with primary human outgrowth supernatants (SupGGR, Figure 24) and primary macrophages (TZM-ggr, Figure 27).

Transmitted/founder virus infection of SupGGR cells

A key feature of any outgrowth platform is the ability to replicate the virus that matters for transmission, persistence, and rebound. The recent discovery of T/F HIV strains has illuminated these genotypes as the HIV that matters in this context, and thus any viable QVOA platform must capture and replicate these strains. To test this in SupGGR, I infected these cultures with a panel of full-length, wild type T/F infectious molecular clones, representing both Clade C (Africa predominant) and Clade B (North America predominant). SupGGR cells were spinoculated for 2 hours in the presence of DEAE-Dextran and plated for culture for 48 hours, followed by

fluorescence imaging and flow cytometry.

All strains of HIV tested in this experiment infected SupGGR cells with varying efficiency, as judged by reporter induction compared to mock-infected cells (Fig. 25). Input virus was not normalized using p24, so these varying efficiencies may represent differences in viral entry or simply differences in infectious titer of each stock. Interestingly, GFP-positive SupGGR cells exhibited distinct phenotypes among the viruses studied. Several, but not all, viruses induced formation of multinucleate syncytia, a finding described previously for the SupT1/CCR5 parental cell line after infection with several human isolates as well as lab strains LAI, BaL, and MCV[135]. Notably, THRO.C, a Clade B T/F clone infected 84% of SupGGR cells after 48 hours, but despite this high penetrance, no significant formation of syncytia was observed. By contrast, ZM249M and CH067.c (Clade C T/F clones) infected many fewer cells overall (12.6% and 7.7%, respectively), but the overwhelming majority of GFP-positive cells in these cultures were aggregated and/or fused with irregular morphology (Fig. 24). A separate Clade C clone, CH162.c infected a much higher fraction of SupGGR cells and also formed small syncytia, though these exhibited a prevailing round phenotype with very few irregularities. While these are descriptive and preliminary findings, they illustrate that viral genotype drives a wide diversity of phenotypes that may impact the entry and exit (budding) of HIV among different cell types. Reporter cells such as MoltGGR and SupGGR provide the means to identify and characterize such phenotypes using viral outgrowth platforms already employed for HIV reservoir studies in human patients.

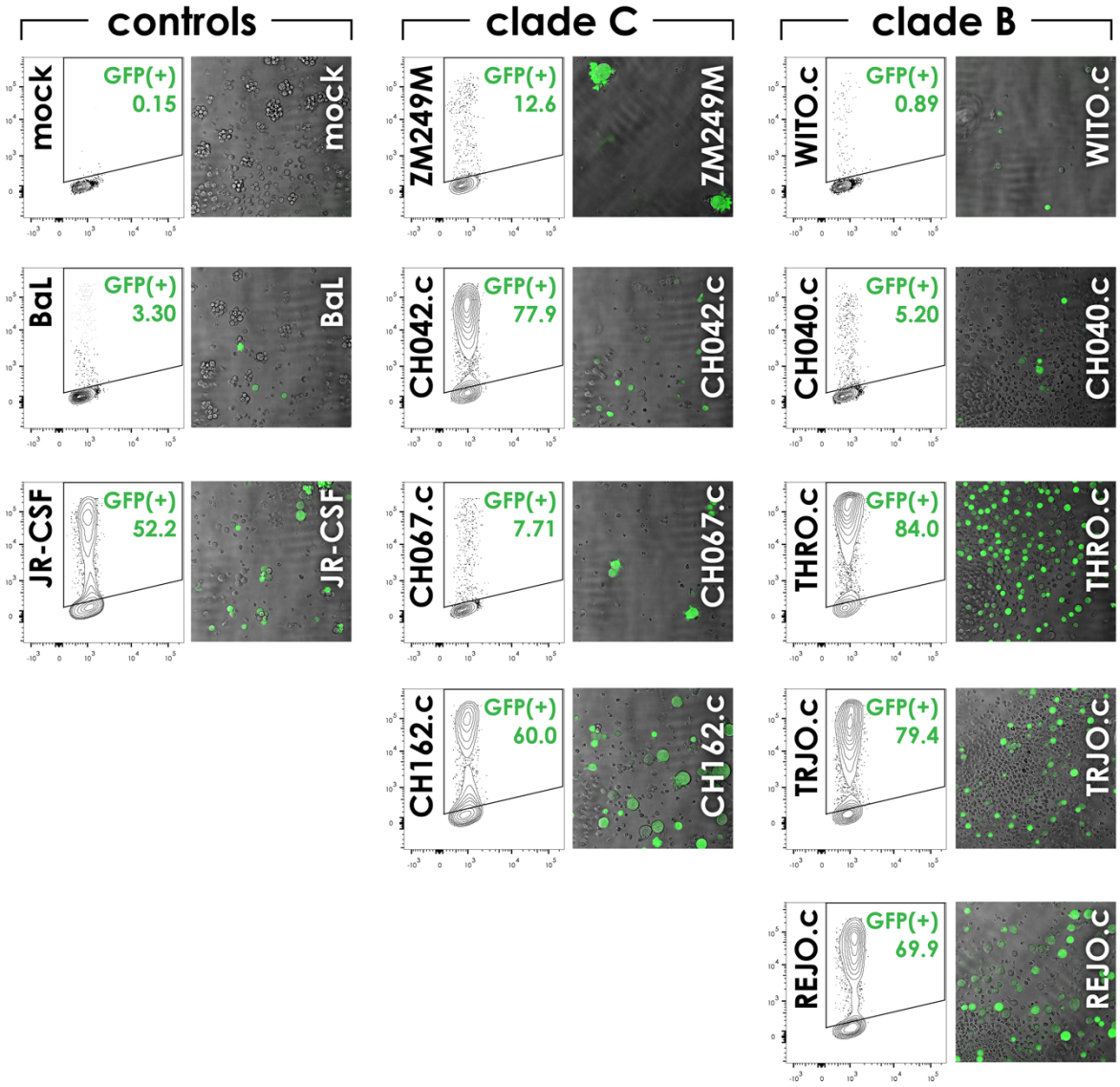


Figure 24. Efficient infection of SupGGR cells (clone F1) with a panel of transmitted/founder HIV strains. Control viruses (left column) were compared with infectious molecular clones of T/F viruses from Clade C (middle column) and Clade B (right column) 48 hours after infection of SupGGR cells. For each virus, representative confocal images are shown with GFP contour plots generated by flow cytometry. Irregular morphology is visible among syncytia and aggregates in Clade C clones ZM249M and CH067.c, with a notable absence of syncytium formation in Clade B clone THRO.c despite the high HIV penetrance in this culture at 48 hours.

Titration of HIV primary transmitted/founder clones in lymphoid (SupT1) vs. myeloid cells (HMDM)

It is well established that T/F clones exhibit poor replication in monolayers of HMDM (monocyte-derived macrophages) in vitro when compared to the expansion of these same clones in PBMCs[58]. In addition, molecular and functional data show the viral envelope of T/F clones is universally CCR5/T-tropic, arguing these strains do not adapt to macrophage infection in vivo[143,145]. These data have been interpreted to strongly suggest that T/F HIV strains are not likely to be found in myeloid cells in vivo, and by extension that macrophages are unlikely to play key roles in the main features of T/F biology: patient to patient transmission and rebound/persistence. One particular study compared p24/gag viral output in the supernatant of CD4 T-cells and HMDM cultures over a period of 10 days, and found that T/F virus production in HMDM was up to two orders of magnitude lower than control lab-adapted macrophage-tropic strains, and 1-3 logs lower than T/F replication in lymphocytes[58]. However, I reasoned that such kinetic analyses omit consideration of the proliferative (lymphocyte) versus static (macrophage) character of each cell type, which would have a logarithmic effect on the results. As a simple example, assuming a conservative 24 hour doubling time, 5,000 lymphoid cells seeded on day one of a ten-day assay would yield $5.1x10^6$ cells (3 orders of magnitude) on day ten, not including any HIV-induced death. By comparison, 5,000 HMDM cells seeded on day one would remain 5,000 cells or fewer by day ten. A more effective measure of T/F HIV biology would address the fitness of these strains at the single cell level. Furthermore, a central tenet of HIV

persistence is the presence of durable HIV infected cells that can seed new infection in new target cells upon interruption of ART. Any estimation of this capacity in macrophages or other cells needs to consider the question of whether a given cell type infected with a T/F HIV strain can productively infect target lymphoid cells and thus give rise to rebound viremia.

I devised an assay to address these questions by comparing the infectious titer of selected HIV clones in HMDM vs SupT1/CCR5 cells. Both of these cell choices are suboptimal surrogates for the cells HIV most readily encounters in vivo, but they are commonly used reagents to model in vivo myeloid and lymphoid HIV infection, respectively. This experiment was designed to establish the absolute titer of a given virus stock in different cell types, calculated as the TCID50[141]. This absolute titer can then be compared among different cell platforms.

It is well known that the infectivity of HIV in different target cells can be influenced by culture additives, tissue culture and inoculation format, and one can tailor the infection to maximize entry of HIV for a given target cell type. Such techniques can make it difficult to compare directly the efficiency of HIV entry across cell platforms. Often used in macrophages, SIV virus-like particles carrying VPX protein increase reverse transcription in myeloid cells[20], a rate limiting step owing to the host restriction factor SAMHD1 that degrades cellular pools of dNTPs in these post-mitotic cells to reduce viral replication[21,22,146]. Antagonizing SAMHD1 leads to dramatic increases in the kinetics and penetrance of HIV infection in

cultured HMDM and other myeloid cultures ex vivo[21,147]. Similarly, cell-free virus infection of lymphoid cells is inefficient in culture[148], and can be augmented by an order of magnitude or greater combining target cells with polycationic reagents such as DEAE-dextran or polybrene, and centrifuging the target cells with HIV in a process called spinoculation. However, none of these features are available in vivo, and a basic comparison of infectious titer drives at the minimal requirements, not maximized infection, of the target cell types. I reasoned that an even playing field without culture or inocula additives was an equitable approach to titer comparison.

To establish the TCID50 of several HIV stocks in cultured SupT1/CCR5 and HMDM cells, serial tenfold dilutions of virus were prepared and added to these cultures in round bottom 96-well dishes. Three 96-well plates were prepared per virus: (1) SupT1/CCR5 @ 2500 per well, (2) HMDM @ 2500 per well, (3) medium only, with 50µL of diluted viral stock added to 8 replicate wells per dilution. Cultures were incubated for five days to allow infections to establish (or for inoculum virus to decay in medium-only wells). On day five, 10^4 reporter SupGGR cells were added to each well to read out replication competent HIV in each well. Using the number of positive wells for each dilution (Fig. 25B), the Reed-Muench method was used to calculate TCID50 in each well type. Background-subtracted TCID50 titers were plotted for each virus in Fig. 25C).

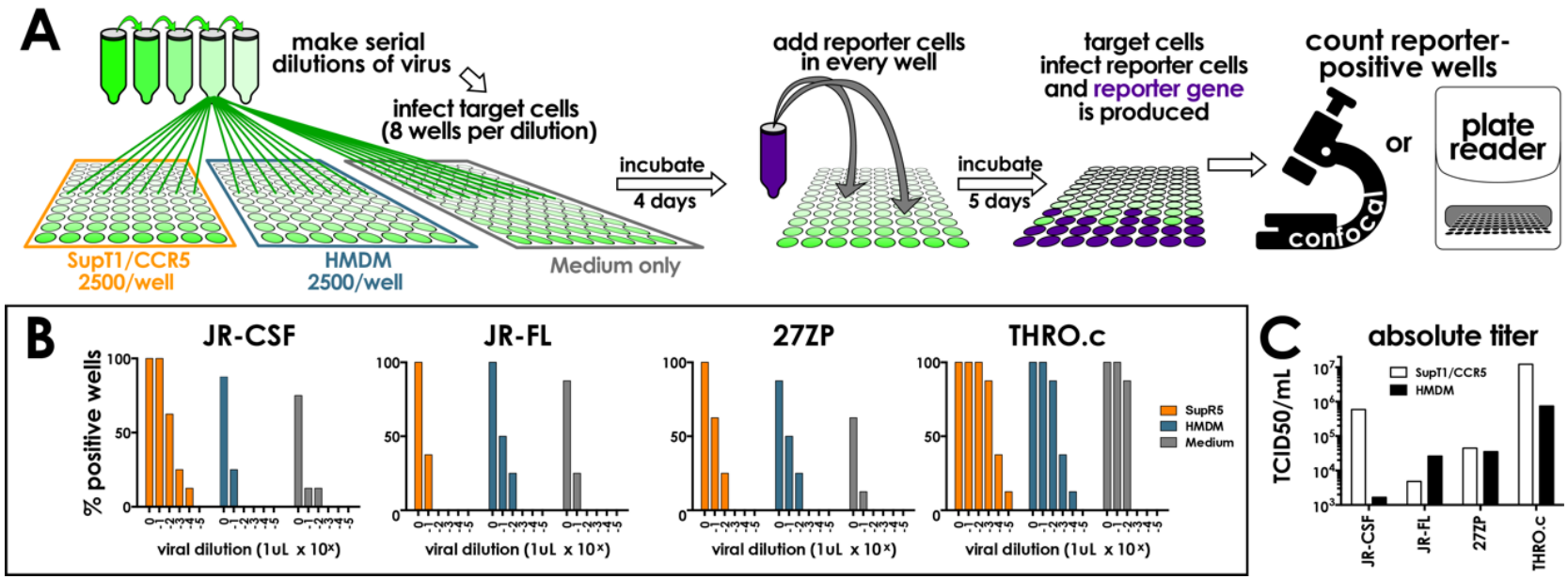


Figure 25. A scalable absolute titer assay measures viral fitness in multiple cell populations. A Schematic of assay setup. The infectious titer of a given viral stock is established in multiple cell types in a single experiment. After the first round (target) infection is established for 4 days, a consistent bolus of reporter cells (here SupGGR) are added to every well, and reporter gene activity used to score wells positive or negative for HIV outgrowth. **B** Plots of percent wells positive vs. viral dilution. These values are used to calculate the TCID50 for each target cell population, and in wells receiving no first-round target cells (medium only). "Medium only" background subtraction then provides the comparison TCID50 in panel **C**, showing that virus 27ZP is equally infectious in lymphoid and myeloid cells, while T/F clone THRO.c is highly infectious in both macrophages and SupGGR cells but significantly more efficient in lymphoid infection.

Control viruses JR-CSF and JR-FL were isolated from the cerebrospinal fluid and frontal lobe, respectively, of an AIDS patient at autopsy, and have become valuable reagents for the study of HIV receptor biology. JR-CSF is a T-cell tropic (CCR5) strain that is considered either very weakly or non-macrophage-tropic[149], while JR-FL is a CCR5-dependent highly macrophage-tropic strain. Interestingly, serial dilutions of these viruses in both SupT1 and HMDM cultures confirmed these existing data, showing a strong preference of JR-CSF for SupT1/CCR5 infection (TCID50 = $5.9x10^5$/mL) versus HMDM infection (TCID50 = $1.7x10^3$/mL), a difference of almost 2.5 logs (Fig. 25C). Conversely, JR-FL was more infectious in macrophages (TCID50 = $2.6x10^4$/mL) than in SupT1/CCR5 cells (TCID50 = $4.8x10^3$/mL). These data validate the assay as an approach to study cellular tropism using full-length infectious molecular clones of HIV.

The human Clade C strain 27ZP was isolated in our lab from PBMC outgrowth of a chronic HIV patient not on ART therapy. This stock is not a cloned reagent but a viral swarm propagated in Molt4R5 cells over 21 days. It has been reported that isolates of Clade C – the subtype of HIV predominant in Africa – use CCR5 as a co-receptor much more efficiently than other HIV subtypes, especially when CD4 levels are low[57], such as on macrophages. The authors found that Clade C envelopes were nearly equally dependent on CD4 and CCR5, while other subtypes were much more strongly dependent on CD4 concentrations on target cells. Here I show that titration of viral stocks of 27ZP – a Clade C virus derived from peripheral blood – revealed approximately equal infectious titer in SupT1/CCR5 (TCID50 = $4.5x10^4$/mL)

and HMDM cells (TCID50 = $3.5x10^4$/mL, Fig. 25C), a finding consistent with previous data but demonstrated using full length infectious HIV.

Finally, I confirmed that the Clade B T/F viral clone THRO.c is more highly infectious in lymphoid SupT1/CCR5 cells (TCID50 = $1.2x10^7$/mL) than in HMDM ($7.4x10^5$) as previously reporter (Fig. 25C)[58]. However, this 16-fold difference in infectious titer does not preclude macrophages as viable targets for T/F infection, since the stock was still highly infectious in HMDM cultures (note that the TCID50 of THRO.c in HMDM was approximately the same as the TCID50 of the T-cell tropic JR-CSF stock in SupT1/CCR5 infection). While preliminary, the data generated here using the absolute titer assay demonstrates a small sample of the many questions that become possible to address with accessible reporter readouts. Future experiments are planned to examine a wider panel of T/F clones for absolute titer in HMDM versus SupT1/CCR5, primary CD4 T-cells and TZM-based cell platforms.

These findings demonstrate that the absolute titer assay uncovers meaningful variations in HIV tropism using infectious clones of HIV. Importantly, while existing assays provide 'cleaner' systems using cloned envelope genes to pseudotype replication-incompetent reporter viruses (a single-round infection assay), these systems can only consider envelope as a determinant of replication fitness in various target cell types, and also bias the results toward HIV strains that excel at cell-free virus transmission. The new model presented here permits flexibility for choices of target cells and outgrowth platforms, including the option to

inoculate multiple different cell formats using the first-round supernatants.

Applications of improved outgrowth platforms to study HIV infection of macrophages

My findings above that T/F clone THRO.c is highly infectious in both macrophages and lymphoblastic cells highlight an important paradigm that has not been adequately addressed in the literature. A bona fide HIV reservoir cell must: (1) be susceptible to HIV infection with relevant strains, (2) survive the infection for long periods during ART therapy, and (3) seed rebound infection upon interruption of ART drugs. So, do macrophage cells qualify as reservoirs? First, while it is clear that T-tropic HIV replication in pure macrophage cultures might be significantly disadvantaged compared to T-cells[58], macrophages do not exist in monoculture in vivo. Indeed, the kinetics of viral replication in a single cell type are irrelevant to the reservoir capacity of the cells in question. I show above that HMDM are highly susceptible to infection with T/F clone THRO.c, despite lower apparent infectivity than in parallel lymphoid cultures. Secondly, I and others have shown that HMDM infected with HIV persist for long periods in culture, while mice harboring human myeloid (but no lymphoid) cells can support viral replication, viremia, and demonstrate persistence of HIV during ART therapy[54,55,95]. And finally, using HMDM models in vitro, it is well established that macrophages not only seed infection in naïve target cells, they do so with a highly efficient mechanism that appears highly targeted and evolved[119,123,125,127,130]. However, while macrophages surpass all the basic requirements for a reservoir cell, robust data remains scarce pertaining to HIV persistence within macrophages in vivo.

For this reason, the HIV macrophage question has been debated for the last 30 years, even as our understanding of the monocyte-macrophage lineage has matured[66-68]. Recent reports from multiple groups have shown that tissue macrophages such as lung AMs are not derived from adult bone marrow macrophages as was long assumed, but are seeded by human fetal liver cells during embryonic development and around parturition, and can persist through the life of the organism without substantial contribution from circulating monocytes. Implicit in this observation is that tissue macrophages like AMs are self-renewing, capable of proliferation in response to mitogenic stimuli in their environment[71]. For AMs in particular, it has been shown in mice and humans that GM-CSF is a necessary factor for the maturation of AMs in the lung[66,68,103], while GM-CSF signaling is required for the proliferation of AMs in situ and ex vivo[150-156].

These findings have enormous ramifications for the study of HIV persistence. IT has been known since the early days of HIV research that primary macrophages (HMDM) infected with HIV *ex vivo* remain viable for weeks, continually producing virus with a reported peak between 40-60 days[95]; compare this with an average half-life of 48 hours for HIV-infected lymphoblasts. While it would be difficult to quantitatively compare the sum total virion production in a single infected macrophage vs. a single infected lymphocyte over their respective lifetime, only infected T-lymphocytes are known to have a finite half-life once productively HIV-infected[46]. The added possibility that HIV infected macrophages might also

proliferate in vivo raises the possibility of clonal expansion of virus without exposure to ART drugs or immune pressure, a concept now being intensely explored even in lymphocyte subsets[157,158]. Finally, the biology of HIV infection in macrophages appears highly evolved to maximize the efficiency of cell-to-cell spread during virological synapse, a process that has been quantified, and imaged in real time[119]. Macrophages may form a self-renewing, long-lived, and consistent source of infectious virus that is not cleared by the immune system or by HIV cytopathic effects. However, despite their seemingly ideal candidacy as long-lived HIV reservoir cells, it remains unresolved whether or to what extent they contribute to viral rebound, persistence, or the HIV latent reservoir.

These are key paradigms to study, but the generally poor access to tissue macrophages from live HIV-infected patients, and the perceived challenges of studying this diverse and complex set of immune cells ex vivo, have together impeded widespread entry of investigators into this arena. Furthermore, the HMDM platform may not be a relevant model for tissue-resident macrophages. HMDM are a terminally differentiated, reactionary cell population with no proliferative capacity ex vivo, while tissue macrophages like AMs are self-renewing and generally exhibit an anti-inflammatory phenotype. This is a fertile area of research, but a systematic approach in pursuit of a better model of tissue macrophage HIV infection has not yet been reported. Here, I explore the potential of fetal liver progenitors as a model of tissue-resident macrophages during HIV infection.

Comparison of monocyte-derived and fetal-liver macrophage HIV infection

HMDM are generated by slow differentiation from adult blood bone marrow-derived monocytes in medium containing 10% human pooled AB serum. During a one week differentiation period, the rounded blood monocytes gradually adhere, transiently assume a spindle shape, then eventually a canonical "fried egg" morphology (Fig. 26B). Proliferation of HMDM cultures during this timeframe is absent. By contrast, fetal liver CD34+ progenitors are cultured for 2-3 weeks in the same human serum-containing medium, but with added recombinant human GM-CSF at 100ng/mL. Their morphology is blastic for the first 1.5-2 weeks, after which the formation of aggregates and progressively adherent foci is observed, during a period of intense proliferation. Eventually, this cell division plateaus and after 3-4 passages in vitro, the cultures become static. Interestingly, mouse fetal liver cells cultured under similar conditions are reported to proliferate indefinitely without transformation[151], a scenario I was unable to reproduce with the human cultures. At 3 weeks of culture human fetal liver-derived macrophages (HuFLM) were post-mitotic, morphologically indistinguishable from HMDM (Fig. 26AB), and were advanced for further analyses.

I first tested the susceptibility of HuFLM to HIV infection under the same conditions as infection in HMDM. Equal numbers of HMDM and HuFLM were seeded for HIV infection and could not be distinguished by morphology upon adherence (Fig. 26AB). However, these outward phenotypic similarities concealed a significant functional difference related to HIV susceptibility. Six days following infection with a

macrophage tropic (BaL) enveloped GFP reporter virus (HIV-GFP, Fig. 4), HuFLM exhibited a threefold increase in HIV-GFP penetrance compared to an equal number of HMDM (~16.6% HuFLM vs ~5.4% HMDM, Fig. 26CD). This effect was reproducible, observed in three separate experiments performed on different days. These data indicate that HuFLM cells are more permissive hosts for HIV infection in vitro. While factors influencing this outcome are unknown, it is possible that dNTP levels in HuFLM remain higher than in HMDM owing to their more recent history of cell proliferation during in vitro differentiation. However, the effect was likely not directly due to proliferating HuFLM at the time of infection, as no differences in PCNA (proliferating cell nuclear antigen) staining were observed between HMDM and HuFLM. Future analyses should investigate the hypothesis that low SAMHD1 expression and the consequent higher residual dNTP levels s in HuFLM cells might contribute to their increased efficiency of HIV infection in culture.

Importantly, cultured HuFLM represent the same lineage that seeds the diversity of tissue-resident macrophages[66-68], and may serve as a more accurate model of their biology than do HMDM. The increased susceptibility of these embryonic-lineage cells to HIV infection is an interesting, reproducible preliminary finding that indicates a divergent host/pathogen interface in HMDM versus HuFLM. However, much more work is needed to redefine in HuFLM some of the paradigms established so firmly in the literature using HMDM, notably pertaining to T/F virus replication and HIV persistence; these features of myeloid HIV infection may be underestimated in HMDMs.

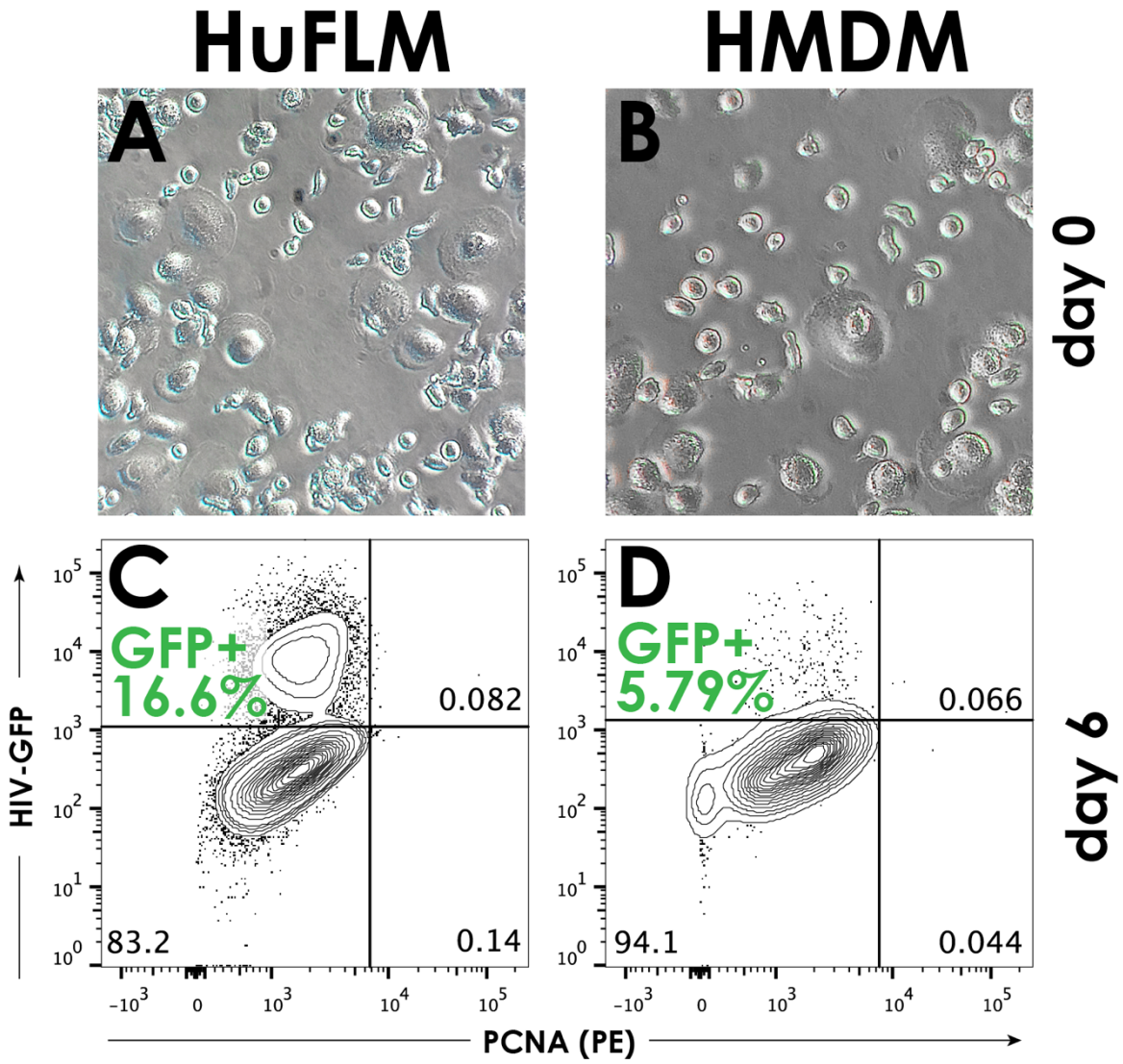


Figure 26. Human fetal liver-derived macrophages (HuFLM) are more susceptible hosts for HIV infection than HMDM. **A-B** Cultured HuFLM and HMDM are morphologically equivalent at the time of HIV infection with HIV-GFP (BaL env). **C-D** On day six, HuFLM (**C**) show a threefold higher penetrance of HIV infection than HMDM (**D**), an effect that did not correlate with any changes in expression of cell cycle antigen PCNA.

Potential of fetal liver macrophages as HIV reservoirs

The increased infection of HuFLM with HIV compared to parallel cultures of

HMDM raised the possibility that HuFLM and their in vivo tissue macrophage progeny might be likely targets for HIV infection in vivo. However, the relevance of these cells as HIV reservoirs would be impacted by their ability to then productively infect target cells during rebound in vivo or co-culture in vitro. To address this, I designed a limiting dilution viral outgrowth assay using lymphoid SupT1/CCR5 cells, and myeloid HMDM and HuFLM infected with VSV/G-pseudotyped HIV-mCherry. The objective was to quantify efficiency of HIV transfer from each cell type to different outgrowth platforms (Affinofile-GGR cells, and the TZM family of cell reagents).

Based on the previously observed robust infection of HuFLM cells with HIV, I hypothesized that these cells might be similarly enhanced in their ability to harbor and transmit the virus to new cells. To normalize for infection efficiency, HIV-mCherry positive cells (SupT1/CCR5, HMDM or HuFLM) were harvested from culture and FACS purified on a BioRad S3e instrument. The sort was straightforward and demonstrates a clear proof of concept for flow- and FACS-mediated experiments with live primary human macrophages. The plots of sorted cells are shown in Fig. 27 B-D, again showing a much higher (~4-fold) higher HIV penetrance in HuFLM versus HMDM. Interestingly, this experiment suggested the difference in susceptibility is not due to HIV env-dependent entry, since VSV/G-pseudotyped HIV-mCherry was used for the initial infection. Sorted mCherry-positive cells were gently centrifuged and resuspended at a concentration of 3-5 cells per 50µL of medium. To initiate co-culture, a total of 50µL (3-5 cells) was then aliquoted into multiple replicate wells containing monolayers of target reporter cells and the cells incubated overnight.

Two days later, wells were inspected for the presence of viable HIV-mCherry-infected macrophages and the development of reporter signal by confocal microscopy. In all wells seeded with macrophages (HMDM or HuFLM), between one and four adherent mCherry-positive cells were found per well. Robust m-Cherry and GFP-positive syncytia were found in Affinofile-GGR wells with co-cultured HMDM and SupT1/CCR5 cells, but surprisingly no syncytia or GFP-expressing foci were observed in wells with HuFLM. In HMDM wells, mCherry-expressing macrophages were evident immediately adjacent to the majority of multi-nucleate syncytia formed, suggesting these cells initiated the infection locally. This observation is consistent with my previous results suggesting macrophages initiate but do not themselves join HIV-induced syncytia in TZM-gfp reporter cells. By contrast, HuFLM cells positive for HIV-mCherry were evident neighboring susceptible Affinofile-GGR cells, with no apparent transfer of infection (Fig. 27 B-C), an absence of reporter signal, and absence of any cell fusion events. These findings were reproduced in wells of TZM-bl and TZM-GGR reporter cells (Fig. 27 E-F), showing dramatic mCherry-positive syncytia developing with an adjacent m-Cherry positive HMDM cell of typical morphology, while no infection events were observed surrounding adhered HuFLM in corresponding wells . Twenty-four hours later (day 3 of co-culture), syncytia in HMDM and SupT1 co-cultures continued to expand in size and fluorescence, with no changes apparent in HuFLM cultures despite the persistence of mCherry-positive cells of typical macrophage morphology. Together the data argue that, despite strong HIV transcription, infected fetal liver macrophages do not produce infectious

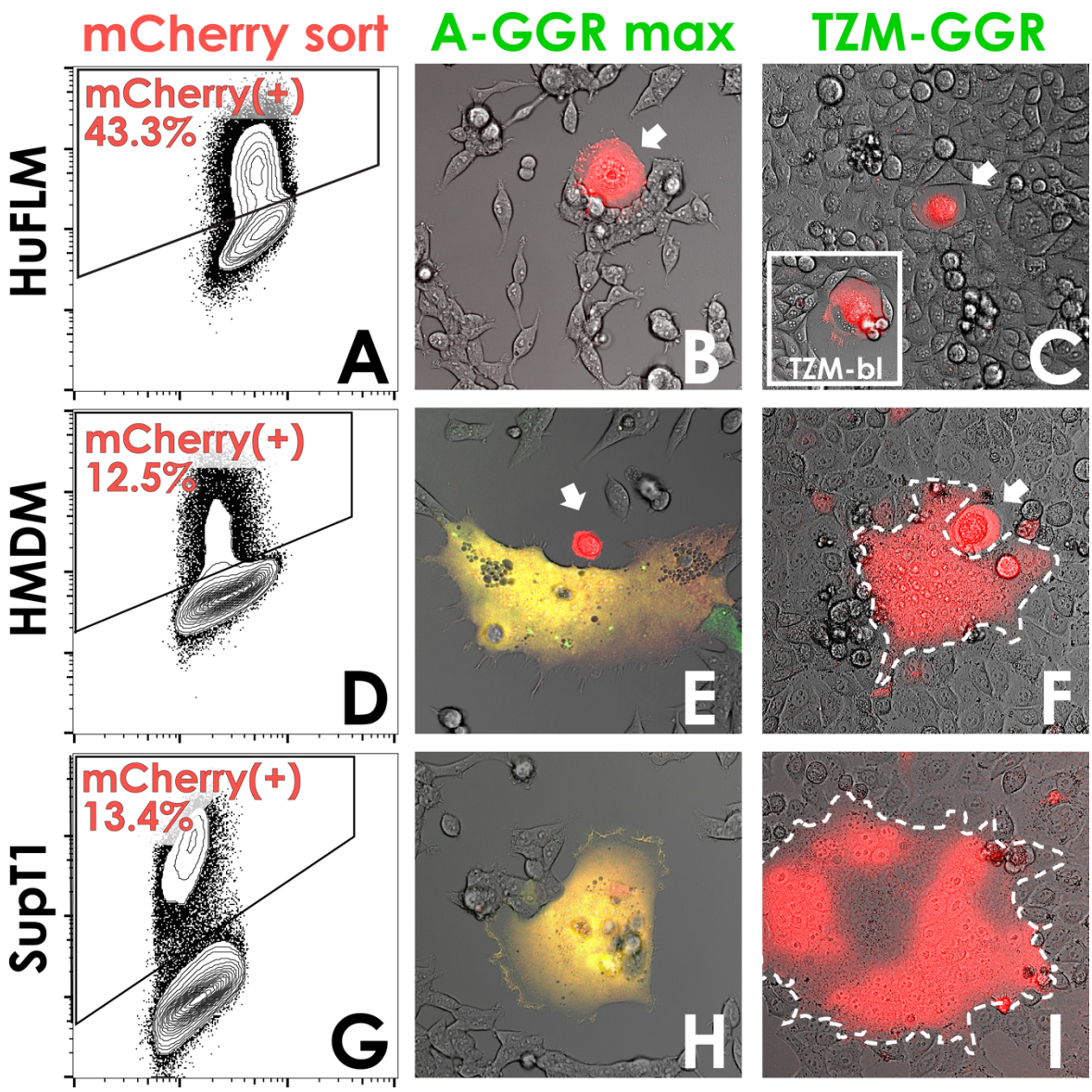


Figure 27. HIV-infected HuFLM do not infect co-cultured reporter cells. A,D,G HuFLM, HMDM and SupTI/CCR5 cells were sorted for mCherry positivity after infection VSV/G-HIV-mCherry (BaL env). Cells were diluted and plated at ~3-5 cells per well in 96-well format with pre-seeded reporter cells (indicated in green). Cultures were imaged 48 hours later (above) and followed 5 additional days. **B** HuFLM do not seed outgrowth in maximally-induced Affinofile-GGR (A-GGR max) or (**B**) TZM-GGR cells. This observation is conserved in TZM-bl cells (**inset, B**). By contrast, mCherry-positive HMDM (**C-D**) and SupT1/R5 cells (**E-F**) seed robust infection in both cell formats. As previously observed for HMDM, and for HuFLM in A-B, infected HMDM adhere, initiate syncytium formation in reporter cells nearby, but do not themselves form heterotypic fusions (**white arrows**).

HIV virions during co-culture with engineered reporter cells. These are highly surprising findings, and strongly argue for a host factor that restricts HIV maturation or release from HuFLM cells under the tested conditions. This would be the first report, to my knowledge, of absolute inhibition of virion production or release from primary cells infected with HIV. While tetherin and the TIM family of Ig domain proteins have been shown to partially restrict HIV release in CD4 T-cells and HMDMs[159-161], the restriction in HuFLM appears absolute. Future experiments are planned to attempt modulation of this pathway to address these and other relevant questions surrounding HuFLM cells and HIV transmission (See Chapter 5).

These are preliminary findings that will require more investigation, without which only very conservative conclusions can be drawn. However, they highlight the large gaps in our knowledge of HIV infection in primary myeloid cells, and importantly provide several novel tools to rigorously and conveniently address the key questions. I describe here several important phenotypes observed through the use of fluorescent HIV reporter cells that are scalable to single cell resolution and flexible with multiple readouts.

DISCUSSION

The rationale for these studies was to illuminate what we might be missing in standard outgrowth assays of HIV in vitro. If a culture well is PCR- or HIV gag (p24) negative at the time of the assay, does that mean no HIV infection was present?

Pioneers in the field based and refined their assays on replication kinetics of CXCR4-tropic late-stage HIV from the peripheral blood, and thousands of papers published on the subject are variations on that theme. Standard cell lines were updated relatively recently to permit the reliable replication of CCR5-tropic virus, the strains that predominate at every stage of infection before and during ART in humans[162,163]. But what if the HIV in tissue macrophages replicates very slowly in vitro? What if the virus is present but remains undetectable by enzymatic assays at canonical timepoints? The tools available to address these questions were designed and validated using a very narrow range of HIV strains, and lack any capacity to provide live metrics of infection if attempting outgrowth of diverse strains from novel reservoirs. Such experiments would be more likely to succeed if endpoints or workflow could be adjusted based on a live readout, especially where input material was limiting and derived by semi-invasive methods such as bronchoscopy and lavage.

To address these challenges, I modified several standard cell platforms with various fluorescent reporter constructs. I show that properties of the industry standard parental cell lines impact their utility under various conditions, bearing important ramifications for experimental design especially in the context of rare infection events, such as serial dilution outgrowth assays. My work highlights the long list of questions that remain unresolved with regard to discrete subsets of reservoir cells in vivo, the HIV they may harbor, and provides several robust tools to study them ex vivo.

CHAPTER FOUR

Application of viral outgrowth assays to characterize HIV infection of BAL-derived human alveolar macrophages

This chapter details efforts to deploy some of the tools developed above in viral outgrowth platforms in Blantyre, Malawi. Importantly, while I originally envisioned the workflow of reporter-informed outgrowth at the clinic in Blantyre, I played just one of many critical roles of this multi-center, multi-investigator study. It has been the honor of my nearly 15-year career in science to participate in a study of such gravitas with direct connection to human patients – an honor experienced by few investigators and even fewer graduate students.

The experiments described in this chapter are part of a collaborative undertaking with members of the Russell, Mwandumba, and Jambo laboratories, In Ithaca, NY, Liverpool, UK, and Blantyre, Malawi. All primary co-culture experiments were performed by others, on the ground in Malawi. I performed all secondary culture experiments and fluorescence microscopy studies. Molecular analyses were carried out by Saikat Boliar, with some of my own contributions.

INTRODUCTION

Since its emergence in 1980 HIV has become perhaps the most intensely studied virus around the world, and for much of the past 30 years has accounted for the largest number of deaths from any single global pathogen[164]. Sustained efforts to bring antiretroviral therapy (ART) to a majority of infected people worldwide – with a focus on sub-Saharan Africa – have provided steady reductions in the number of HIV-related deaths. Paradoxically however, a corresponding decrease in the number of new infections has stalled in the same timeframe[164]. In 2016, only half of the HIV-infected population around the world were receiving ART, showing we still have a long way to go.

Blantyre, Malawi is a city near the geographic epicenter of the HIV epidemic, in a country faced with more than 9% HIV prevalence among adults[165]. Among HIV-infected individuals in Malawi taking frontline antiretroviral therapy (ART), each year approximately 10-15% experience ART failure, underscoring the importance of understanding reservoir cells and the virus they harbor. While many investigators globally have pursued latent HIV reservoirs in bloodborne CD4 T-cells, tissue reservoirs are much more difficult to study. Tissue access is limited, often surgical, and medical justification for such studies is challenging. Notable exceptions are routine diagnostic procedures such as BAL and colonoscopy, and are being pursued in our lab and elsewhere to access potential reservoir tissues for the collection and analyses of HIV-infected cells[74]. The high coincidence of active pulmonary tuberculosis (TB) with HIV infection in sub-Saharan Africa means that in

Malawi, BAL is a diagnostic standard of care. In this procedure, AMs and other cells are washed out of the airway and collected for diagnosis of tuberculosis and other opportunistic pathogens. Blantyre is an ideal setting for such studies, and Dr. Henry Mwandumba has been a highly valued collaborator of our laboratory for many years in this context. Indeed, Dr. Mwandumba and our laboratory first developed the FISH:FLOW platform as a means to detect HIV infection at the cellular level during what was originally a TB-centric study of AM function[74].

Quantitative studies of Clade C reservoirs

Industry standard methods to quantify the HIV reservoir are labor-intensive and costly; hence, much of the quantitative work on HIV reservoirs has been done in a handful of laboratories, in large part on North American patient cohorts (Clade B HIV)[166]. As a consequence, very little is known about the size and character of HIV reservoirs in African (Clade C) cohorts, where HIV genotypic differences are known to drive key phenotypes particularly related to virus entry and baseline macrophage tropism[57]. The current study is an early step toward addressing this shortage, and is designed to provide reliable, industry standard tools to researchers in a wide variety of settings (including resource-limited) with the means to at least illuminate what we might be missing using common viral outgrowth assays.

RESULTS

Primary co-culture for HIV capture in Blantyre, Malawi

Previous work from our lab used FISH:FLOW and functional assays to show

that HIV gag-positive AMs were found preferentially in the 'small' AM subset (low forward scatter) and were deficient in phagocytic activity[74]. However, HIV RNA FISH signal has not yet been reliably associated with replication competent HIV infection in AMs. We hypothesized it was feasible to capture infectious HIV provirus within primary co-cultured cells and supernatants of AMs, TZM-gfp or Molt4R5 cells. In parallel we plated adherent co-cultures of TZM-gfp and AM cells on coverslips, for analyses by electron microscopy. The result was a coordinated workflow that is manageable in the context of a busy patient recruitment and bronchoscopy schedule in Malawi, under the direction of Dr. Henry Mwandumba. The primary culture window (48 h) was short in compliance with institutional biosafety requirements (the risk of TB outgrowth in primary cultures precludes long-term BSL-2 experiments).

Bulk cells from BAL including AMs, lymphocytes and other cells were plated with reporter TZM-gfp cells or non-reporter Molt4R5 cells in culture for 48 hours of primary culture. In some cases, patient-matched PBMC were isolated and seeded separately in co-culture for viral capture. In theory, this window would allow for a single round of HIV infection only, and would result in shedding of the virus into the medium of infected wells. The magnitude of virus production would be dependent on the number of primary BAL cells productively infected. Cells were harvested either using trypsin-EDTA for adherent TZM-gfp or by centrifugation for Molt4R5 cells. In both cases, primary culture supernatants were saved for additional outgrowth, vRNA and HIV p24 quantification. Cells were viably archived in DMSO-containing

medium, and both cells and their associated supernatants were forwarded to Ithaca, NY on dry ice for downstream analyses.

Analyses of HIV content within primary co-culture supernatants

Primary co-culture supernatants from ten patients were first analyzed by p24 ELISA (Fig. 28) to test for the presence of viral gag antigen. This assay is an industry standard estimate of HIV virion concentration in culture medium or patient-derived material. It is not used as a surrogate for infectious HIV, since defective virions and cellular debris contain gag protein. Of twenty samples analyzed (multiple co-culture formats were plated for some patients), we detected p24 antigen in two samples from different patients, 10.52M (BAL) and 10.27Z (PBMC). Both p24-postive samples derived from TZM-gfp co-cultures; the TZM-gfp outgrowth platform was not the only successful strategy however, as infectious HIV-1 was later found in supernatants deriving from Molt4R5 co-cultures (described below). Interestingly, patient 1052M is an ART-treated individual (duration >1 year), who at the time of BAL harvest had a CD4 count of 471, and an undetectable plasma viral load.

The PBMCs of patient 10.27Z (ART naïve) also yielded p24-positive supernatant in TZM-gfp co-culture, but only after 48 hours of culture. The 24-hour timepoint could not be distinguished from background p24 levels. These findings suggest either that infection of TZM-gfp cells produced new virions that were detected only on the second day of culture, or that primary cells shedding virions did not accumulate sufficient p24 in the medium by 24-hours to be detected by the

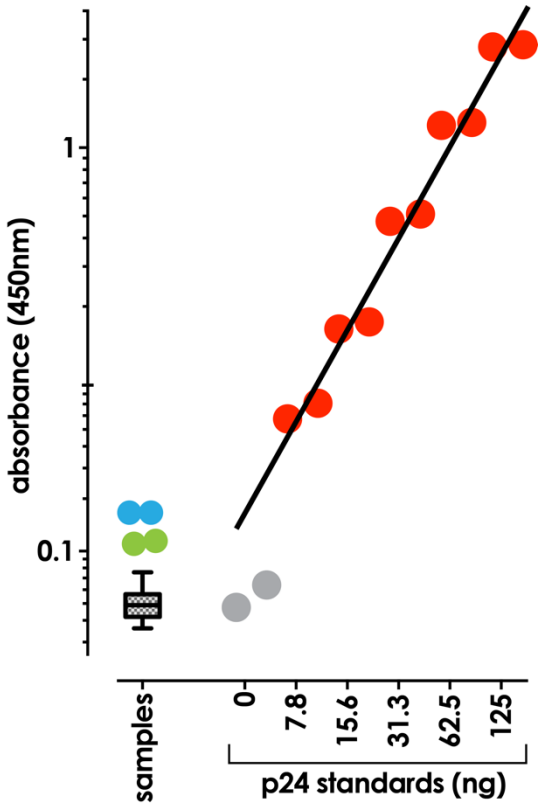


Figure 28. Measurement of HIV-1 p24 concentration in primary co-culture supernatants from Blantyre, Malawi. A series of p24 standards (red) demonstrate the linear region of the assay. Pooled samples (hatched box) are plotted with outliers established using the Tukey method of 1.5x IQR. Positive samples plotted correspond to patient 1052.M (ART positive, green) and 10.27Z-PBMC (ART naïve, blue).

assay. Demonstration of budding HIV by electron microscopy, or secondary outgrowth culture assays would be required to establish the replication competency of the HIV we detected.

The detection of HIV p24 in primary culture supernatants from an ART-treated patient was important preliminary evidence obtained using an industry-standard assay that HIV is present in the airway of human patients. Importantly, these results

were obtained without exogenous cytokines stimulation and in 48 hours of primary culture, arguing that active, not latently-infected cells seeded outgrowth or produced virions in the assay timeframe. However, more rigorous proof of infectivity and the genetic sequence of the virus would be needed to demonstrate that HIV in the airway has reservoir potential in the context of ART.

Primary co-cultures harbor HIV virions revealed by electron microscopy

TZM-gfp co-cultures with primary BAL were performed on coverslips in Malawi, that were fixed and transported to Ithaca, NY for processing and electron microscopy (EM). Similar to the schematic performed using correlative EM in Figure 18, regions of interest were embedded in resin and the coverslip removed prior to ultrathin sectioning in the z-axis.

Alveolar macrophages and many HeLa/TZM-gfp cells were observed by EM imaging. While accumulation of HIV within VCCs of cultured AMs was not observed in the samples examined, characteristic virions of HIV morphology were found in several samples deriving from BAL co-culture (Fig. 29), including from patient 10.27Z, 10.55G and 10.53K. No patients on ART regimens were examined by EM in this pilot study. However, the relative ease of HIV identification by EM in these samples despite no robust downstream outgrowth of virus from secondary cultures of 10.55G and 10.53K validates this combined approach to collect evidence of HIV production within cells of the airway, and increases confidence that low-level or rare signal observed during secondary outgrowth in Affinofile-GGR or Molt4R5 cells

corresponds to virus that does not meet the burden of fitness for replication in vitro under current conditions. Future experiments should revisit these methods to support outgrowth data obtained from ART treated patient samples.

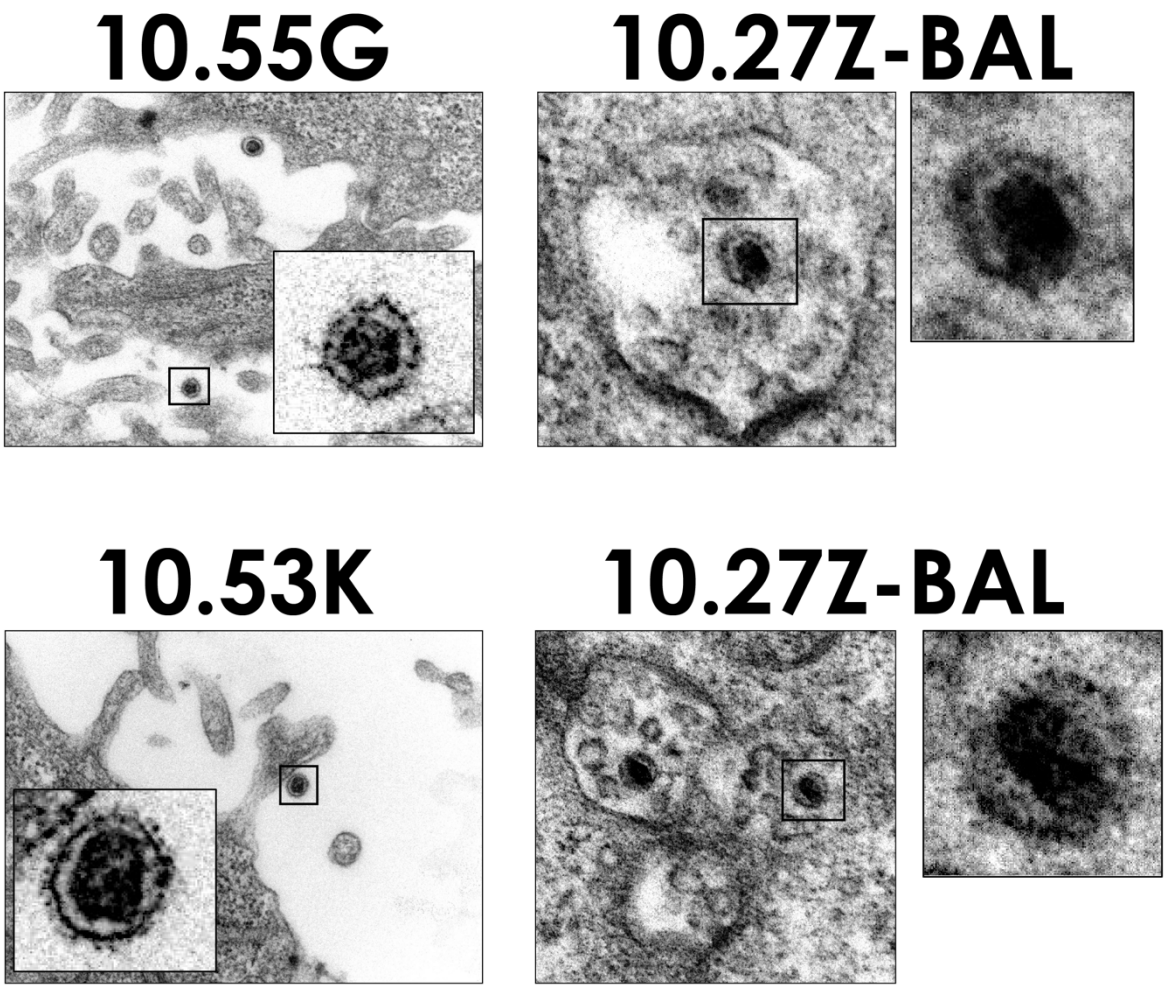


Figure 29. Primary BAL co-cultures reveal HIV virions budding in TZM-gfp cells. Cultures from three patients not on ART were analyzed by electron microscopy. HIV was evident throughout the culture, with representative fields shown.

Secondary outgrowth cultures: primary cells reseeded for outgrowth

Our working hypothesis held that harvested co-culture cells would be suitable hosts to transport the virus and would provide outgrowth in secondary co-cultures at Cornell University. Encouraged that some primary supernatants were p24-positive with evidence of virion production by EM, we thawed cells for a longer-duration secondary co-culture with either Molt4R5 cells or maximally-induced Affinofile-GGR cells ($CCR5^{hi}/CD4^{hi}$). Cultures were propagated for 10 days, and Affinofile-GGR (A-GGR) cells were monitored daily by fluorescence microscopy for the emergence of GFP-positive foci that might indicate active HIV infection. These efforts were unsuccessful however, and we were unable to generate any evidence of HIV replication in these secondary co-cultures. PCR on isolated vRNA from the secondary co-culture medium and amplification of A-GGR or Molt4R5 cellular RNA produced no HIV-derived amplicons in any sample tested, even those that had returned a positive HIV p24 result in earlier tests (Fig. 28).

Secondary outgrowth cultures: supernatant inocula

Parallel cultures using primary co-culture supernatants as inocula were more successful. Molt4R5 and maximally induced A-GGR cells were used as secondary outgrowth targets for these supernatants, and cultures were carried for 13 days

post-inoculation. Daily monitoring of A-GGR cells revealed the emergence of rare, early GFP-positive foci by 42 hours (not shown), with syncytium formation characteristic of HIV cytopathic effects by 3 days post-infection (Fig. 30). Compared to robust infections observed using positive control lab-adapted HIV strains (Fig. 30, top row), patient-derived supernatants exhibited much-reduced apparent infectivity, with very rare (2-10) GFP-positive foci per 35mm dish (except in mock-infected cultures receiving no inoculum). By day 4.5 however, it was clear the number and size of most GFP-positive foci were not expanding logarithmically (with the exception of those originating from PBMC-derived primary co-cultures).

Affinofile cells were chosen in these experiments due to the rapid and highly robust readout following HIV infection with all viral strains previously tested. However, these cells require the addition of drugs to culture medium to induce the co-receptors for HIV. Thus, one possible explanation for the initial signal that appeared to slow after 2-3 days in culture is a decline in HIV co-receptor expression (CCR5 and CD4) in the absence of induction by doxycycline and ponasterone-A. Hence, on day 5 cultures were dosed with new induction drugs, incubated 2 additional days to allow a single round of infection with virions already present in the medium, then passaged on day 7 onto freshly-seeded and induced A-GGR cells. However, robustly expanding infection was confined only to cultures derived from patient sample 10.27Z-PBMC.

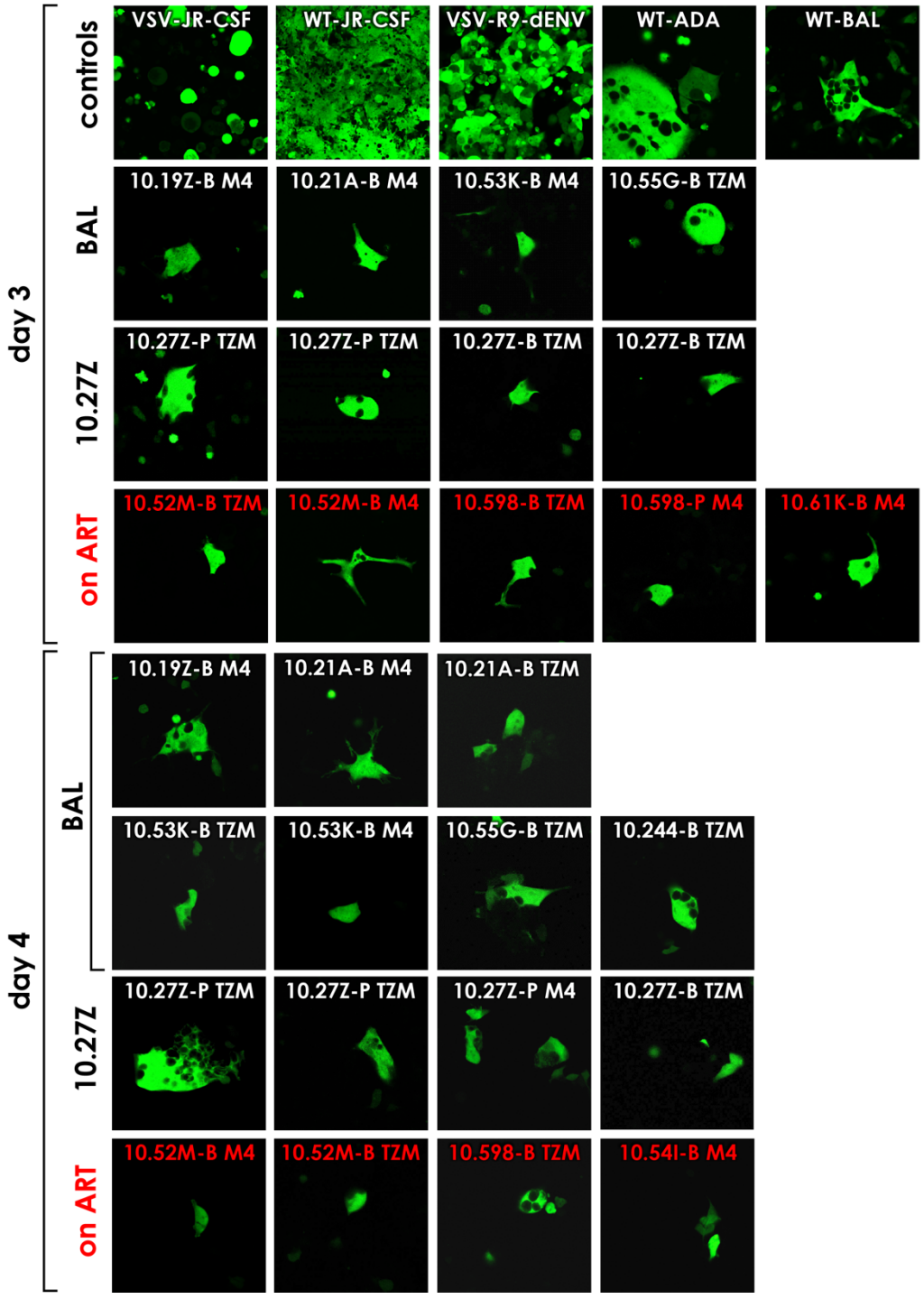


Figure 30. Live confocal imaging of HIV outgrowth cultures in Affinofile-GGR cells. Primary viral supernatants were added maximally-induced A-GGR cells and cultures were images on day 3 (**top panel**) and day 4 (**bottom panel**) post-inoculation. Samples from ART-treated patients marked with **red**. PBMC (**-P suffix**) and BAL (**-B suffix**) samples from Molt4R5 (**M4**) and **TZM**-gfp capture were tested. Cultures developed hallmarks of HIV infection in A-GGR cells, but persistent syncytia did not expand beyond day 4, except 10.27Z-PBMC.

These findings seemed to suggest the presence of HIV replication in several cultures of A-GGR cells early post-inoculation, but that replication was not sustainable under the assay conditions tested. Given the sensitivity of the A-GGR readout and observed characteristic morphology of HIV infected Affinofile-GGR cells, cultures were incubated for 6 additional days in an effort to allow viral amplification. However, foci were progressively lost with no additional evidence of replicating infection. The apparently self-limiting nature of the infection was unexpected in this culture format, but was a scenario that could not be adequately modeled *a priori* using lab-adapted virus. This wait-and-see approach of a more prolonged culture was unsuccessful, and PCR on both supernatant vRNA and cell-derived mRNA harvested at 13 days yielded no HIV-1 amplicons, with the exception of 10.27Z-PBMC.

Lymphoid cell platforms generate evidence of viral outgrowth

Interestingly, in contrast to the secondary outgrowth cultures in Affinofile-GGR cells, a greater subset of the same supernatants inoculated into Molt4R5 cells did produce PCR-detectable HIV infection by day 13, assayed by vRNA PCR. These cultures were spinoculated every other day for the first week to maximize HIV spread. These samples included BAL-derived medium from 10.27Z and 10.244 primary co-cultures, and PBMC-derived medium from 10.27Z cultures. Among these PCR-positive cultures, however, HIV replication was markedly attenuated compared to lab strains of virus. When assayed for HIV by removing a small sample of supernatant and testing infectivity on A-GGR cells, only laboratory strains BaL and JR-CSF and 10.27Z-PBMC cultures produced infection (not shown). Moreover,

attempted expansion in Molt4R5 cells of BAL-derived virus to generate infectious stocks was unsuccessful, suggesting these viruses adapted too slowly to Molt4R5 culture conditions and were eventually lost. This stands in contrast to the outgrowth of 10.27Z-PBMC-derived HIV, which replicated to high titer in Molt4R5 cells and generated infectious stocks of 5 x 10^4 TDIC50/mL, a titer comparable to that of HIV-1 BaL viral supernatant prepared by transient transfection of 293FT cells. Importantly, no cultures of ART-treated patient-derived material replicated in this assay format sufficient for PCR amplification in either the supernatant or cell lysate. Ultimately, Molt4R5 outgrowth yielded multiple PCR-derived sequences from patient 10.27Z (BAL and PBMC) and 10.244 (BAL), covering the full length ORFs for *env*, *tat/rev* and *gag* (Fig. 33).

As others have previously reported[167], our findings may reflect the poor infectivity of some primary HIV isolates in vitro, which may not be adaptable to transformed cell lines in our assay timeframe. The sensitive readout of the reporter cells I describe here indicates that these tools may identify early infection events missed by standard measures of bulk viral replication, and afford opportunities to study the mutations associated with adaptation to culture over time. Interestingly, while we were able to isolate the genetic sequence of the 10.27Z (BAL) virus from Molt4R5 cells, replicating virus stock could not be obtained in the Molt4 platform even after extended culture, despite the strong outgrowth of patient-matched 10.27Z (PBMC) stocks in the same cells.

The SupT1/R5 platform supports replication of HIV isolates that other cell lines do not

Whereas primary TZM-gfp or Molt4R5 co-culture supernatants from 10.27Z (BAL) failed to generate a replicating infection during secondary outgrowth in Molt4R5 or A-GGR cells, inoculating these same primary supernatants in the SupGFP platform gave rise to robust replication (Fig. 31). These primary supernatants had originally tested negative by p24 ELISA, and produced only a self-limiting infection in Molt4R5 cells that was sufficient to clone some viral sequences by PCR, but not to generate infectious stocks. By contrast, outgrowth in SupGFP cells was continuous and logarithmic, with the Molt4R5 primary supernatant apparently replicating more efficiently than that from TZM-gfp primary co-cultures (Fig. 31). Viral stocks isolated from both samples at 21 days reflected this advantage, with Molt4R5 stocks

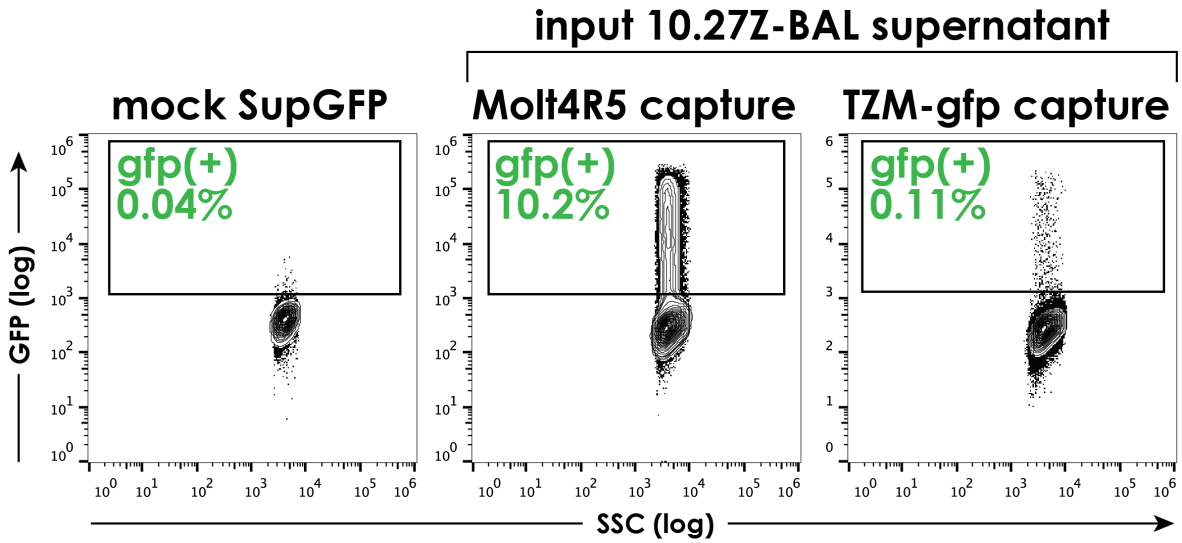


Figure 31. SupGGR cells support replication of HIV from BAL-derived supernatant. Compared to mock-infected SupGFP cells (**mock, left panel**), cultures receiving 10.27Z-BAL supernatant from the Molt4R5 (**middle panel**) or TZM-gfp capture platform (**right panel**) reveal robust induction of GFP reporter. These cultures generated infectious stocks and yielded PCR env clones after a 21-day outgrowth period.

exhibiting a nearly fifteen-fold enrichment in HIV titer (TCID50 in TZM-bl, data not shown). These data show the variability of HIV primary isolate fitness in various transformed cell populations, and help validate the approach of testing multiple outgrowth platforms with accessible readouts.

Envelope evolution in different in vivo compartments and during outgrowth in vitro

Envelope clones were generated from patient 10.27Z to compare the full-length sequences of virus replicating in the peripheral blood versus the airway, and to learn whether the choice of capture platform (TZM-gfp vs. Molt4R5 primary co-culture in Malawi) or outgrowth platform (Molt4R5 vs. SupGFP secondary outgrowth in Ithaca, NY). Figure 32AB shows the schematic of cell isolation. Three separate secondary outgrowth cultures were initiated from BAL-derived supernatants and one for PBMC-derived supernatant. Env clone sequences were aligned and clustered (PhyML, Figure 32C). Interestingly, this revealed that the outgrowth cell line (Molt4R5 or SupGFP) impacted viral adaptation more than the capture cell line, a finding that may be a consequence of the much longer secondary culture window (21 days vs. 2 days for the primary capture window). Viruses adapting to culture conditions would converge based on pressures from the host cells over this extended timeframe. However, tissue origin also impacted the clustering of these envelope genes, as the coding mutations apparent in PBMC vs BAL-derived cultures centered heavily in the V3-V5 loop region, with special emphasis in the V4 loop of env ectodomain. Importantly, a high fraction (~29%) of the sequence differences between PBMC and BAL-derived virus disrupted residues known to be

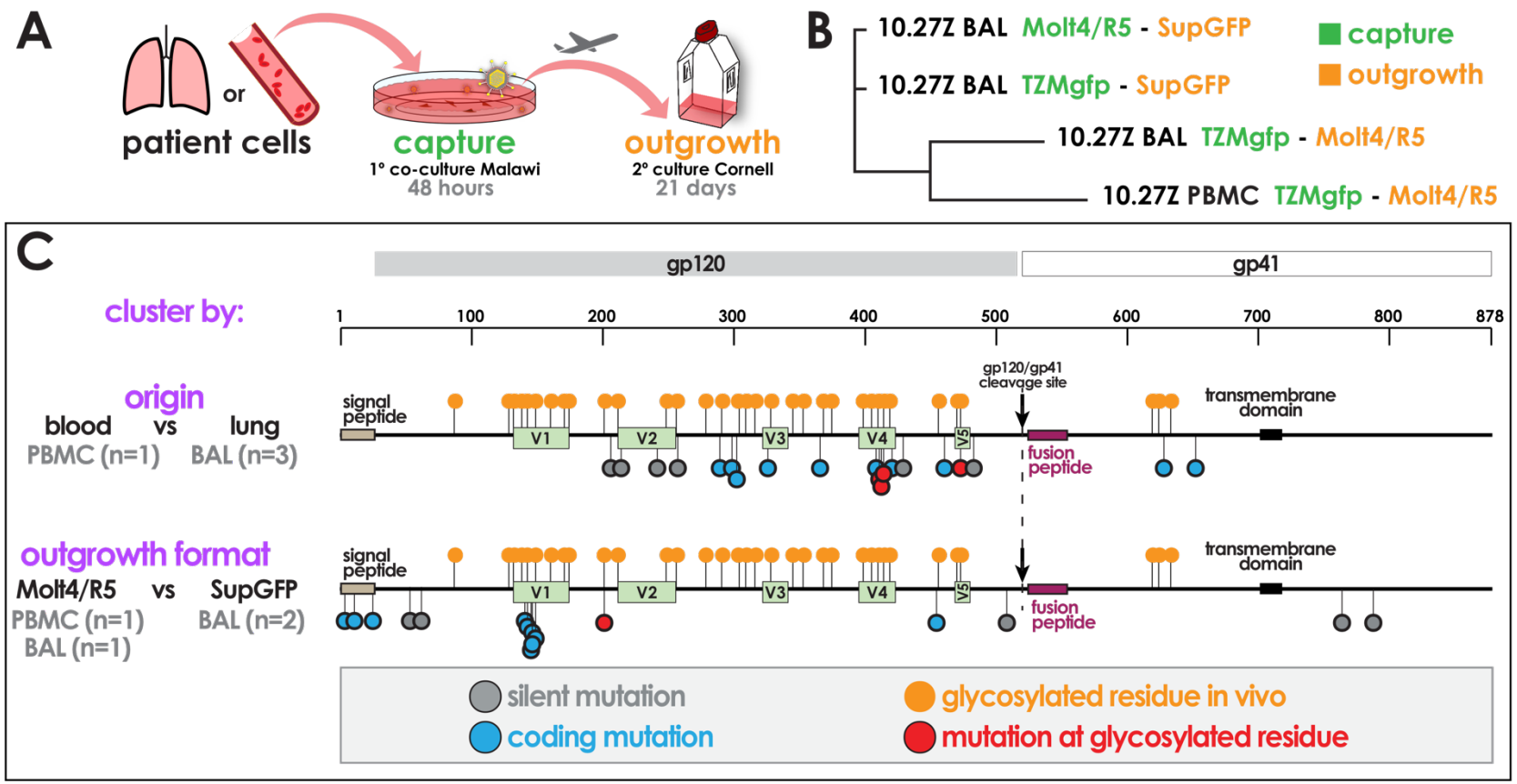


Figure 32. HIV isolates from a single patient reveal anatomic compartmentalization and in vitro adaptation dependent on outgrowth platform. **A** Supernatants were transferred to Ithaca, NY for secondary outgrowth culture at Cornell University. **B** Phylogenetic clustering showing that 21-day HIV outgrowth cultures in SupGFP (**orange**) cells are more closely related than supernatants derived from the same capture format (green TZM-gfp). However, three samples deriving from BAL cluster together. **C** Non-uniform accumulation of mutations dependent on host pressure. Comparing mutations arising in PBMC vs BAL samples (**origin, purple, top genome panel**), most coding mutations (**blue**) appear in V4 and V5, with 29% of coding mutations disrupting N-linked glycosylation (**red residues** have disruptions, all glycosylated residues highlighted with **orange**). Comparing mutations in Molt4R5 vs. SupGFP outgrowth format, the majority of mutated residues fall in the signal peptide and V1 loop. Silent mutations denoted by **grey** markers.

N-linked glycosylated in vivo[168]. By contrast, the V1 loop and signal peptide seemed to dominate among mutations that clustered according to outgrowth cell line (Molt4R5 vs SupGFP) with comparatively little impact on the glycan shield (9% of coding mutations). These data suggest divergent pressures on the virus in vitro during outgrowth and in vivo during adaptation to different anatomical compartments. Importantly, very few sequence differences were identified within the V3 loop among these clones in accordance with previous findings[169].

A summary flowchart of the culture formats and outgrowth results is given in Figure 33. The workflow validates a combined approach to attempt viral outgrowth, and has refined our goals for subsequent cohorts. The primary conclusion from these studies was the marked success of primary outgrowth supernatants in subsequent downstream analyses versus work on viably archived primary co-culture cells. Our future pursuits will feature longer primary co-culture windows of five days to allow viral expansion prior to supernatant harvest, combined with the use of the SupGFP cell platform for primary outgrowth in Malawi to easily monitor outgrowth by flow cytometry with limited liquid handling and convenient longitudinal sampling.

DISCUSSION

The viral outgrowth platform in Blantyre, Malawi is still being refined. With the tools I have developed, team members in Malawi and Ithaca have advanced the program with critical improvements at each iteration. We have shaped the protocol for viral capture and characterization around hard-won data, but have

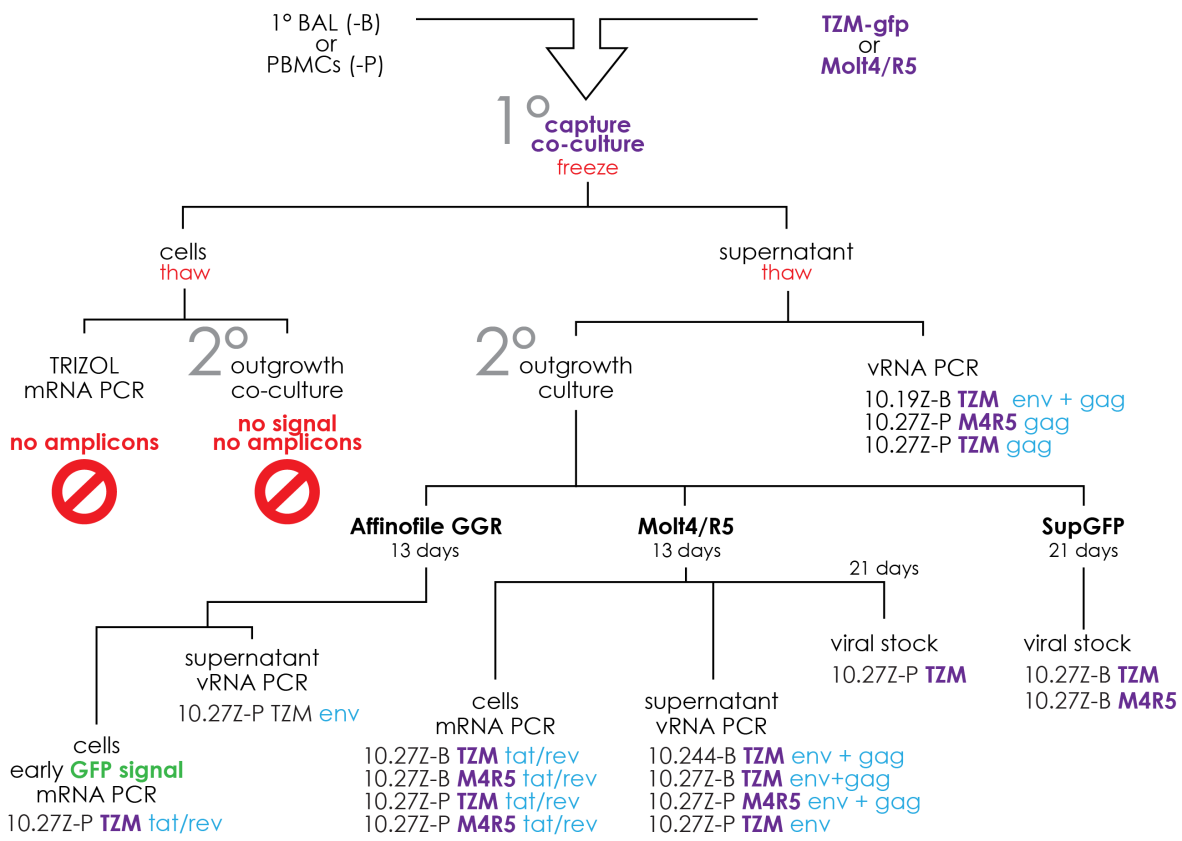


Figure 33. Flowchart summary of reporter cell-guided viral outgrowth results. Primary BAL cells (**-B suffix**) or PBMCs (**-P suffix**) were co-cultured in Blantyre with capture cell lines TZM-gfp or Molt4R5 (capture format indicated in **purple**). Secondary outgrowth culture was performed in Ithaca, NY. Successful PCR and viral output is indicated for each sample including specific genes amplified and sequence verified shown in blue.

much more work to do. This type of project is of course bigger than any one team member's contribution, but my concepts and cellular reagents have made iteration possible through rapid, convenient readouts at critical timepoints of the protocol. Using TZM-gfp, SupGGR, and Molt4-based platforms, we have produced large viral swarms from both BAL and peripheral blood, which can now be characterized at the single-virion level using variations on the assays I present here. Future experiments should seek to understand any differences or compartmentalization of the viruses in the peripheral blood compared to the

airway, their molecular and cellular tropism using both affinity profiling (Affinofile cells) and absolute titer experiments, and characterize the heterogeneity in each vial population for clones that are either strongly or poorly adapted to replication in vitro.

Furthermore, while our previous data showed that HIV gag mRNA FISH signal is found more commonly in AMs than lymphocytes in the airway[74], the contribution of lymphocyte-derived HIV in these outgrowth assays cannot be excluded. With a more reliable and matured primary outgrowth platform being tested, future experiments should address the cellular source of any HIV outgrowth from human BAL (see Chapter 5). Such pursuits were considered premature for the current studies prior to proof-of-concept, but would entail immunomagnetic separation of myeloid from lymphoid cells in BAL samples. Nonetheless, rigorous demonstration of HIV replication in the airway of Malawian adults, absent information about the specific cell types harboring the virus, was considered to be a significant advance that would help define our future directions in Blantyre, and clarify the role of the airway as an anatomical reservoir for the virus.

CHAPTER FIVE

Future work: broadly expanding horizons

In this chapter, I address the challenges and exciting opportunities ahead using the tools and insights generated during these studies. While my work highlights several paradigms that need attention, this thesis lays the foundation and provides the infrastructure to make rapid progress towards these goals.

HIV-infected monocyte-derived macrophages are refractory to heterotypic cell fusion

I repeatedly observed that HIV-infected human monocyte-derived macrophages (HMDM) could seed rapid outgrowth of HIV in co-culture with a variety of reporter cell platforms. This was especially true of adherent reporters such as the TZM family of HeLa-based HIV reporter cells, and of HEK293-derived Affinofile-GGR cells (Figures 14-16, 27). Using several lab-adapted and primary isolates, the strength of the reporter cell readout was bolstered by the formation of large, multi-nucleate syncytia that expanded in size and signal intensity over several days in culture. A key feature of such syncytia is an upregulation of HIV output and longer half-life of the fused cells[170], a windfall for outgrowth studies attempting to capture rare HIV-infected cells in a primary cell population. However, the dramatic formation of these cell fusion events was contrasted with the appearance of a solitary adhered HMDM cell in the immediate vicinity, that was observed to be HIV-positive in experiments using fluorescent reporter virus such as HIV-mCherry (Fig. 27). This is correlative data, but the strong suggestion is that an HIV-infected HMDM adhered and transferred the infection locally to adjacent reporter cells, initiating a chain reaction of serial infection and homotypic fusion among the reporter cells. The macrophage itself, however, does not appear to fuse membranes and join the growing syncytium, as previously reported[119].

The fusogenic properties of the HIV envelope protein are well known to induce cell fusion among neighboring cells[171,172]. Indeed, all enveloped viruses

employ a variation on this theme, using envelope/spike-mediated mechanical deformation of the cellular membrane bilayer sufficient to disrupt the hydrophobic interactions among phospholipid tails in both membranes and thereby evert the membranes to mix lipid phases[173]. Conjugate pairs of neighbor cells (one HIV-infected and the other expressing the viral co-receptors) participate in similar membrane fusion, only between two cells instead of a cell/virion pair. However, that macrophages immediately adjacent to receptive cells can transfer the viral infection without engagement in membrane fusion argues against a simplistic model of how HIV is budded or transmitted. It is possible that the fluid properties, lipid or protein content of macrophage plasma membrane are inherently different, such that they are refractory to such deformation. Consistent with this notion, it has been reported that cholesterol-rich lipid raft domains are required for HIV infection in macrophages, suggesting that subdomains with distinct physical or chemical properties are subject to viral membrane fusion[174]. However, dependent on the viral envelope protein, macrophages are themselves susceptible to cell-free HIV infection, proving the macrophage plasma membrane can be bent to accommodate HIV entry. Moreover, macrophages can actively bud large quantities of infectious HIV virions, showing the plasma membrane is competent, somewhere along it's perimeter, for robust HIV exit. While the universality of HIV budding into VCCs has been debated[175,176], multiple groups have suggested that membrane subdomains are highly enriched for viral assembly and budding activity that co-localizes with intracellular compartments enriched for env, gag, tetraspanins, ESCRT machinery, and the restriction factor tetherin, linking virions to

the membrane and to each other[123]. These findings may partially explain the low level of macrophage participation in heterotypic syncyita, since in HMDM cells the viral envelope proteins and co-receptors may be sequestered away from cognate binding partners on adjacent cells, pinched off from the extracellular space into VCCs. The lumen of such compartments is connected to the extracellular space by very thin channels, which prevent viral escape in the absence of a virological synapse[124-126]. One avenue to test this hypothesis is the addition of exogenous ATP to HMDM co-cultures, which causes evacuation of VCCs in HIV-infected macrophages[132]. If sequestration of fusogenic components is driving the absence of HMDM heterotypic fusion, then large scale emptying of these VCCs may expose this machinery to neighbor cells and thus to fusion.

Despite being refractory to cell fusion with other fusogenic cell types however, HMDM do retain the ability to form homotypic syncytia (classically referred to as giant cells), for example demonstrated by the addition of GM-CSF and IL4 to the culture medium[177] (Fig. 35). One possible means to augment the transfer of HIV infection to neighboring cells would involve stimulation of heterotypic fusion among co-cultured HMDM and reporter cells, especially in low-inoculum scenarios such as from ART-treated human samples or rare HIV-infected cell subsets

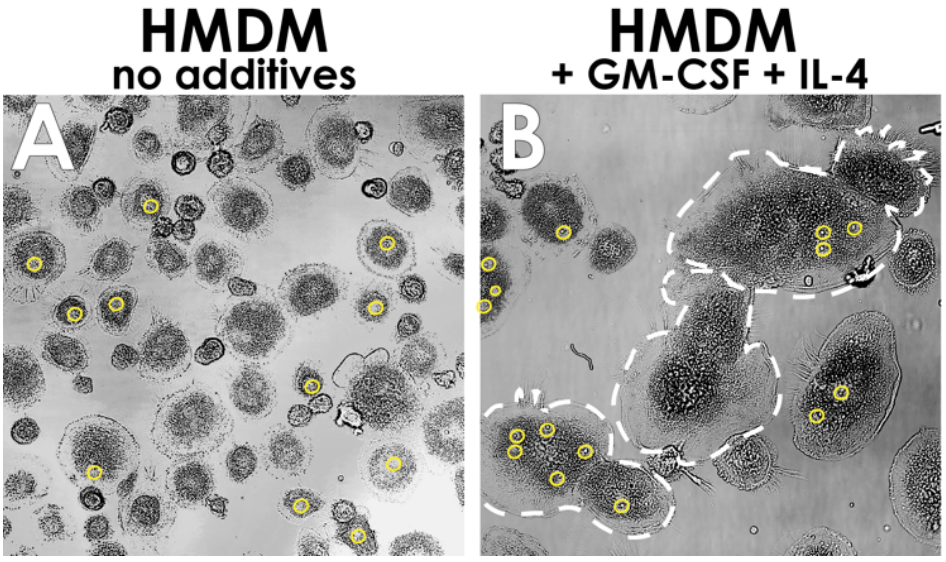


Figure 34. HMDM possess fusogenic capacity. A HMDM cultured for 48 hours in the absence of culture additives exhibit characteristic morphology with one nucleus per cell (yellow circles). **B** Addition of IL-4 and GM-CSF induces formation of multinucleate homotypic syncytia (white dashed boundaries) within 48 hours.

harboring virus of unknown in vitro fitness. As further evidence of specialized regulation of macrophage fusion properties, very recent data revealed that productively infected T-cells can form contacts with cultured HMDM, resulting in cell fusion and binucleation[178]. Interestingly, this initial fusion event further dysregulates HMDM anti-fusogenic properties causing additional cells join the syncytium, reminiscent of those seen in the tissues of HIV- and SIV-positive individuals at necropsy. The absence of this observation using HMDM co-cultured with adherent reporter cell lines (TZM- and Affinofile platforms) might indicate that primary lymphoblasts (and not engineered cells) are required to induce fusogenic behavior, likely downstream of cytokine secretion[178]

Even more interestingly, however, is the notion that the fusogenic tendency of infected macrophages might be governed by the viral sequence (likely env) they harbor – different viruses may cause cell fusion while others do not. This is an important question, as the longevity of a macrophage in vivo as a solitary cell or as a syncytium participant would be different, impacting the persistence of the virus. Fusion activity is determined, in part, by the cytoplasmic domain of gp41, where immature gag polyprotein binds and prevents premature fusion activity before the virus is properly encapsidated and enveloped[179]. And just as there are HIV strains with canonical 'syncytium-inducing' character in primary lymphocytes (mostly CXCR4-tropic strains isolated during late stage AIDS disease), so too might there be strains with varying capacity to induce homotypic fusion among infected macrophages. For example, in very recent preliminary experiments I observed highly divergent phenotypes among T/F viral clones in HMDM, both at the level of infectivity, and in their capacity to induce cell fusion among HMDMs in monoculture. While in previous studies, clone REJO.c was found to replicate in HMDM cultures[58], albeit markedly less than in lymphocytes, I found this clone induces a strong homotypic fusogenic phenotype in HMDM cultures compared to other T/F infections (data not shown). The uncoupling of homotypic fusogenic capacity with replicative potential in various cell types is an interesting paradigm, but what is missing at this point is more robust data to assign cell-cell fusogenic capacity of a given virus in macrophages to a specific env sequence motif. Future studies should address the impact these events have on shedding of HIV and forward infection in naïve T-cells or reporter cell target populations. These properties

may contribute to the way in which certain viral strains transmit between cells, and their biology in macrophages and lymphoid cells requires further characterization.

Using the tools developed here, these questions can be systematically addressed. Using the single cell QVOA platform described in Fig. 22, macrophages (HMDM and HuFLM) and lymphocytes (primary blood lymphocytes and SupGFP cells) should be infected with a large panel of T/F viruses to identify trends that may align with HIV subtype (Clade), tropism, or infectivity in lymphocytes vs. macrophages. To normalize for infectivity of individual stocks, these primary macrophages should be FACS-purified, as in Fig. 27. To obtain fluorescent readout in primary macrophages using wild type T/F strains, the pNL-GFP-RRE(SA) lentiviral reporter should will be used. My recent work has shown this is a feasible approach using HMDM and several T/F viruses, and revealed differential fusogenic properties among the T/F clones in HMDM, notably clone REJO.c. Importantly, very few cells are required for these experiments (50-70 GFP-positive cells per 96-well plate, or ~0.5 cells per well). As shown in Figure 22, positive wells emerge by gaussia luciferase (GLuc) readout very early during the assay, and global differences in the kinetics of GLuc signal development are directly interpreted as varying cell-to-cell infection efficiencies at early timepoints (note the difference between BaL and 27ZP outgrowth at 3 days). Using results from HuFLM in Figure 27 as a guide, similar experiments using single sorted HuFLM cells harboring varying T/F HIV infections would be hypothesized to yield little or no outgrowth in the absence of stimulus to release the virus (discussed further below). This type of experiment is biologically rich,

and depends on the single cell resolution of the input.

This pivotal feature of the platforms I describe stands out considerably from the bulk culture approaches pursued in the field[78,114,135]. Specifically, these standard approaches rely on bulk PCR or enzymatic readouts, which conceal all the nuances that might define the biology of HIV during cellular transmission. Armed with new, more tractable tools, future questions should address whether heterotypic membrane fusion in macrophages and reporter cells is genetically determined by the virus, and how sequence variation drives augmented or handicapped cell-to-cell transfer kinetics. Similarly, using cell-free supernatants from wells seeded with single infected macrophage or lymphoid cells, we need to ask how viral genotype versus source cell type determines the rate of shedding and infectivity of produced virions. With the tools described here, these questions are now approachable.

Systematic analyses of the intracellular virus-containing compartment in macrophages

We and many others have reported the accumulation of mature and maturing virions in plasma membrane-connected VCCs in monocyte-derived macrophages. These structures have been imaged with timelapse microscopy using fluorescently-tagged HIV gag, which is shed *en masse* onto the surface of a synapsing T-cell in culture[119]; these targeted T-cells then become productively infected. Such data are difficult to refute. However, despite the allure of the hypothesis, the extent to which this process is a viral adaptation remains unknown. One possible approach is to ask

whether various viral strains differentially accumulate in VCCs or shed continuously. Indeed, much of the current knowledge has been attained studying a very limited set of lab-adapted macrophage-tropic strains of HIV. Using the correlative electron microscopy approach in Fig. 18, primary HMDM or HuFLM cells can be transduced with the pNL-GFP-RRE(SA) reporter construct to generate a primary reporter "cell line", as I have very recently achieved (not shown). One could also re-engineer the reporter vector to express a bicistronic gag-imCherry ORF in addition to the IRES-GFP cassette to visualize the location of gag protein accumulation in these primary "reporter cells". These macrophage reporters would then be infected in bulk (6-well dishes) with a panel of competent T/F clones, and HIV-positive cells purified by FACS as described above. A single infected cell is plated per well, with target cells added in co-culture, and the fate of virions can be imaged over time. In theory, one could image all 96 wells for several days. In the same experiment, GLuc reporter signal in target cell lines can be measured longitudinally to correlate the timelapse of virion transfer with quantitative productive infection. The outcome is a visual representation of whether or not VCCs are evacuated after exposure to target cells or other modulators (assessed by fluorescently-tagged gag protein), and whether this depends on the viral genotype in question. While there are many variables here, with 96 (or 384) wells on a plate there's room for all the controls. If interesting phenotypes are observed among certain cell-virus pairings, these can be probed more deeply – even by high throughput screens for compounds that modulate VCC evacuation. What if a drug could stop myeloid cells from shedding virus? There are many questions here worth answering.

Alternative hypotheses for lack of HIV transfer from infected HuFLM

This intriguing observation may be due to virion accumulation in VCC, but other explanations are feasible. Virions may be produced but retained at the plasma membrane, or they may not be produced at all. For example, it is known that BST2/tetherin protein physically tethers virions to the plasma membrane surface and prevents their release[97,159,160]. This restriction factor is counteracted by adaptations in the HIV-1 vpu protein to induce effective shedding of HIV virions during infection. Similarly, the T-cell immunoglobulin (Ig) and mucin domain (TIM) proteins also prevent HIV release, notably TIM3 in HMDM[161]. Knockdown of this gene augments virion production from infected HMDM. Interestingly the TIM proteins have also been found to significantly increase HIV entry in macrophages[161], and the possibility that HuFLM overexpress TIM proteins could simultaneously explain their increased susceptibility to HIV infection and their apparent inability to propagate the infection.

I present evidence that HIV-mCherry-positive HuFLM cells do not transmit the infection to co-cultured cells. However, the current data cannot exclude the possibility that virions are entirely absent within HuFLM. Electron microscopy studies currently in process will reveal whether any virions accumulate within VCCs or at the plasma membrane of HuFLM in culture. In case no formed virions are observed, RNA FISH:FLOW experiments and qPCR using gag probes will establish whether gag mRNA is present.

Finally, recent findings show that in HMDM in vitro and AMs in vivo, high endogenous levels of dUTP produce uracilated proviral genomes that yield hypermutations in offspring viruses[96]. While it is clear in HuFLM cells that the LTR promoter and the mCherry ORF are sufficiently intact to drive transcription, the remainder of the genome may harbor mutations that interfere with virion production. Follow up studies should address this explanation for failed transmission.

Whatever the mechanism, this important observation warrants further investigation. The potential of novel restriction factors or post-transcriptional regulation of HIV production will yield important insight into the behavior of HIV within myeloid cells, and may represent pathways specific to tissue-resident macrophage reservoirs in vivo.

Mechanisms of increased HuFLM susceptibility to HIV

The observed increased susceptibility of HuFLM to HIV infection is a novel finding, given the controversy surrounding the efficiency of HIV infection in macrophages in vitro (modeled by HMDM). However, the mechanism for this feature in HuFLM remains unexplored. While I present evidence that cell proliferation is likely not a factor in this observation (Fig. 26), the recent history of proliferative potential in these cells may be important. For example, the pools of dNTPs used for cell division in HuFLM may be governed by expression of SAMHD1 as in HMDM. Despite their exit from the cell cycle, dNTP levels may not have been suppressed in these cells.

Comparison should be made between HuFLM and HMDM for expression of SAMHD1 and whether knockdown or overexpression experiments can modulate HuFLM susceptibility to infection. Several known HIV restriction factors were discovered in this way, comparing RNA expression profiles of restricted vs unrestricted cells.

Modeling alveolar macrophage biology with fetal liver-derived macrophages in vitro.

It is known that tissue macrophages such as AMs, microglia, and Kupffer cells (liver) derive from an embryonically-derived precursor wave that initiates in the yolk sac, is specified in the fetal liver, and populates the tissues in late gestation or early post-natal period[66-68]. These tissue macrophages are self-renewing, and proliferate to replace their ranks in the case of genotoxic insult[66,68,180]. In the lung, the differentiation and self-renewal of AMs is dependent upon GM-CSF signaling, without which AM precursors are present but non-functional and short lived[68,154,156]. Interestingly, this property is shared with HuFLM, which rely on GM-CSF during culture for both differentiation and proliferation. Furthermore, in humans and mice with bone marrow ablation and transplant, AMs are still recipient-derived a year post-transplant[68-70,156], showing the longevity of these cells that derive from the earliest days of the embryo. Conversely, in lungs that have been transplanted into a recipient human, the AMs are still donor derived months or years after the transplant, arguing that these cells can persist for a large majority of the life of the organism[181]. By contrast human monocyte derived macrophages never divide in culture, and are short lived in vivo[182]. These cells are recruited to areas of infection

or tissue injury and responsible for inflammatory cascades and wound healing. Yet despite these large differences in the role and phenotypes of HMDM and tissue macrophages in vivo, we model virtually all aspects of myeloid infection biology in monocyte derived macrophages (true in humans and mice).

I have shown that large functional differences also exist between HMDMs and HuFLM pertaining to HIV infection (Fig. 26-27). Given these disparities, I hypothesize that HMDM may be a misleading model of tissue macrophage biology for HIV studies. At the very least, we should seek to resolve whether the observed differences in HIV infection between these two cell types can be correlated with their underlying biology. To address this, a series of foundation experiments will be very useful. The performance in QVOA assays of naïve AMs from uninfected volunteers should be compared with that of HMDM and HuFLM. Do AMs "cluster" more closely with HMDM or HuFLM? Furthermore, the heterogeneity of AMs should be addressed at least on a cursory basis with immunomagnetic separation of AMs from interstitial macrophages[183] that may also appear in the airway, especially in the context of disease. Basic assays of macrophage function, including those our lab previously reported[74] can be used to align the phagocytic, proteolytic, and oxidative function of AMs with HMDM or HuFLM. Moreover, the transcriptional profile of HMDM, HuFLM, and AM should be compared in the presence or absence of HIV infection to learn the global response of these cells types to infection.

Finally, the observation that HIV release or maturation is delayed or inhibited in

infected HuFLM cells should be probed in AMs as well, given that viral outgrowth assays from AM cells have been challenging in our hands and in those of several other groups. It is attractive to speculate that tissue macrophages (modeled by HuFLM) may accumulate virus in the absence of a proper inductive signal to release it. Perhaps activated lymphocytes are sufficient for such a signal – the simplest version of release regulated by the most advantageous target cell of the virus. However, tissue-specific signals may also transact in this process, a paradigm much more difficult to study.

Together, the existing data suggest functional divergence in HMDM and HuFLM cells[67,70,180]. Whether HuFLM cells are truly a better model of tissue resident macrophages remains unknown, but their lineage and observed proliferative potential argue they share important phenotypes that pertain to HIV infection. Nevertheless, the notion that studies of HIV biology in macrophages may be poorly modeled in HMDM remains a possibility. The accessibility of human AMs, HuFLMs, or other primary macrophage populations such as those from colonoscopy tissue biopsies, make this an important and feasible question to resolve.

Define the boundaries of novel QVOA platforms

I reported that differences in gaussia luciferase (GLuc) reporter activity were observed between BaL and 27ZP strains at 3 days of co-culture in the single cell QVOA platform (Fig. 22), and that for 27ZP, no additional sensitivity was gained after

8 days. However, differences in replication fitness of various strains studied and the cell formats employed will impact these timelines. What is needed is a more comprehensive test of the assay timepoints using a panel of viruses, with reporter sampling daily. The value of these types of readouts is their improvement over the current p24 ELISA or qPCR readouts to allow HIV determination in minutes, and in real time. However, trends for each cell format using several viruses are needed as a training set to guide their deployment in human cohorts. We are currently collaborating with Dr. Andrew Lever's group in Cambridge, UK to test the SupGGR platform in their clinical pipeline. More studies will be needed to establish the utility of the SupGGR and other cellular tools reporter here in diverse applications and cohorts.

Absolute titer of transmitted founder HIV in myeloid vs lymphoid cells

Data showing poor replication of T/F clones in HMDM cells are interpreted to mean that macrophages likely play no role in their biology in vivo. However, the specific titration experiments I describe here reveal that, while one such clone is ~16-fold more infectious in lymphoid (SupGFP) vs myeloid cells (HMDM), the viral stock was highly infectious in both cell types. In the context of high viral titer during acute HIV infection in vivo (10^7 copies per mL of blood), and the dominance of T/F clones during the key stages of infection (transmission, acute, and rebound), a 16-fold reduction in infection efficiency of macrophages may be of little practical significance. On the balance of these observations, I expect that many macrophage cell types in diverse tissues of the body are productively infected with

very relevant strains of T/F HIV, and that these macrophages play key roles in persistence and transmission. Consider for example the airway: AMs comprise about 80-95% of all leukocytes recovered in BAL fluid. During acute infection, exposure of AMs to high titers of T/F HIV in the blood or migrating lymphoid cells would almost certainly lead to their infection; AM longevity and durability during HIV infection would make these cells key candidates as reservoirs. Furthermore, the airway is not the only myeloid-dominant compartment. The brain is an immune-privileged site with an overwhelming majority of microglia and other macrophage cells compared to lymphoid subsets[51,147], and has been well studied in the context of HIV infection and compartmentalization[53,55,69,98]. Moreover, just as the airway has direct access to the outside environment, so does the seminal vesicle and the testes. Like the airway, the dominant leukocyte fraction in the seminal fluid are macrophages, and have been demonstrated to harbor a high burden of cell-associated HIV even in the relative absence of cell-free virus[184,185]. Thus, while slight handicaps in T/F HIV infection of macrophages are observed in vitro, the likelihood remains high that macrophages are key targets for infection in vivo and may contribute to persistence and transmission. Finally, what macrophages might lack in terms of susceptibility to T/F virus infection, they make up with longevity and increased efficiency of transmission to target cells[97].

Optimized primary HIV outgrowth pipeline in Blantyre, Malawi

Future studies in Malawi will focus in the near term on extending the primary culture window to allow additional rounds of replication prior to supernatant

harvest. With our work over the last several years, the pipeline for these clinical samples has dramatically improved. We have obtained replication-competent virus in primary and secondary outgrowth experiments sufficient to clone full length env and gag genes, and in one case have patient-matched infectious viral stocks from both lavage and peripheral blood. Preliminary functional studies have been performed on a limited set of envelope clones, with the goal of establishing their HIV co-receptor usage (CD4 vs CCR5 titration). Using an infectious viral swarm isolated from the peripheral blood of a patient not on ART I show that Clade C circulating virus is equally infectious in macrophages and lymphoblastic cells, underscoring the urgency with which macrophage reservoirs for HIV in sub-Saharan Africa remain to be characterized. Using additional samples in this pipeline we aim to expand on these results and report trends in the tropism or anatomic compartmentalization of Clade C virus in Malawian adults in the presence and absence of ART. Virus genotype vs titer in FLM; evidence for adaptions?

Cellular source of HIV in the human airway

Of utmost importance in reservoir studies is not the presence of virus in a specific anatomic compartment, but the cells in which this virus persists during therapy. A central controversy in the field is whether macrophages can harbor HIV in vivo. Anecdotal evidence suggests infectious HIV is found in macrophages of the airway[101,102,105,106,108], and our lab has shown the presence of HIV RNA in AMs and their consequent functional deficits in phagocytosis[74]. However, the studies

reported here perform outgrowth on bulk BAL cells, which include mostly macrophages but also some lymphocytes. Thus, whether macrophages are the origin of viral outgrowth in this platform has not been definitively shown.

Two main approaches will help demonstrate the cell of origin for HIV in the airway. With the co-culture pipeline in Malawi now established, physical separation of the cell subsets is possible to separately interrogate myeloid and non-myeloid subsets for outgrowth of HIV, from patients treated or untreated with ART. This first approach would involve immunomagnetic separation of bulk BAL with CD3 and CD33 microbeads to enrich lymphoid and myeloid cells, respectively. While long established protocols to harvest primary macrophages involve their purification by rapid adherence in cell culture[186,187], more definitive evidence requires cell surface identification of lymphocytes and monocytes. The sequential positive selection of CD3-positive lymphocytes, followed by CD33-positive myeloid cells[188,189] would maximize the return of the relatively minor subset of lymphocytes in the airway. Using the QVOA platforms described here, the outgrowth of virus from both enriched subsets could be tested. Importantly, independent of the outgrowth fitness of HIV within either cell subset, the early readouts afforded by the GGR platform reporters I describe make it possible to characterize early isolates from both cellular and supernatant RNA pools, and obtain their genomic sequence.

The second approach for defining the specific cell types harboring HIV in vivo derives from a single cell sequencing platform "SeqWell" developed by Alex Shalek's group at MIT[190]. The system uses custom arrays of nanowells, thousands of small machined cavities on the array surface to hold a single cell and a single bead. The BAL cell suspension is loaded onto the array, and single, dissociated cells settle

into each well with a Poisson distribution. To these wells are added lysis buffer and single magnetic beads, each labeled with unique barcoded oligonucleotides that capture poly-adenylated RNA from the lysed cell. Capture transcripts, including HIV transcripts expressed, are bound and reverse transcribed in the wells while bound to the beads[190]. Once these beads are pooled and the oligos cleaved, the cDNA library contains thousands of barcoded species that can be sequenced and aligned to the human and HIV genome. The barcode of reads aligning to the HIV genome are matched with reads of the host transcriptome that have the same bead barcode, and the cell type harboring HIV can be inferred from the transcripts expressed.

We have performed a pilot experiment using this invaluable technology on the ground in Malawi and have returned the HIV sequences from three separate samples, as a proof-of-concept. Underway now is the analyses of the host transcript and refinement of the platform for follow-up studies.

The intrinsic value of SeqWell is the ability to analyze host transcriptional changes within HIV-infected cells in vivo[190], from a large pool of input cells. Host responses to HIV infection will help illuminate potential targets in diverse cell subsets. It is clear from the persistence of HIV during long term antiretroviral therapy, that targeting specific cellular reservoirs won't follow a 'one size fits all' model.

Concluding Remarks

Persistence of HIV is driven at the cellular level by cell type-intrinsic biology that shapes the production or latency of HIV during ART[39]. Our current drug

regimens attack viral replication at the level of new infections, such as inhibition of HIV entry, protease, integrase, and reverse transcriptase. However, current therapies do not affect reservoir cells already infected with HIV. Much work remains to elucidate the ways HIV interacts with multiple host cell types, so these processes can be specifically targeted with new therapies. The first step in this process is retooling, to illuminate the ways in which novel reservoir cells are different, and find the opportunities we didn't know we were missing.

METHODS

Cells

Cells were obtained through primary repositories ATCC (8E5/LAV) or the NIH AIDS Reagent Program (TZM-bl, CEM, 8E5/LAV, Molt4R5), or through generous gifts from academic investigators (JC53, David Kabat, OHSU; Affinofile-GGR, Benhur Lee, Mount Sinai; SupT1/R5, James Hoxie, UPenn). 293FT cells were purchased from Invitrogen.

Cell culture

HMDM were generated from elutriated monocytes obtained from the University of Nebraska Medical Center. Monocytes were plated at 1 x 106 / cm2 culture area in various culture formats, in human macrophage medium: DMEM medium containing 2x L-glutamine, 1X sodium pyruvate, HEPES, 1x Pen/Strep, and 10% pooled human AB-type serum (SeraCare). Cultures were incubated for 7 days, with complete medium being supplemented by 30% v/v/ 2 and 5 days after plating. Medium was changed at 7 days, and cultures were fed three times per week thereafter until harvest or infection.

For experiments requiring reseeding, HMDM were cultured on untreated bacteriological plasticware. HMDM were harvested by washing with DMEM and 5 minute treatment with Tryple-Select (Invitrogen) followed by gentle scraping with a rubber policeman and inactivation in serum-containing medium. Cells were

centrifuged in conical tubes at 200 RCF (1000 RPM) for 5 minutes at room temperature. Medium was discarded and cells resuspended and counted for replating. Under these conditions, cell viability was high and morphology unchanged after reseeding.

HuFLM were generated from culture of 5 x 105 CD34+ human fetal liver progenitors (Lonza) in human macrophage medium described above with added recombinant human GM-CSF (Peprotech) at 100ng/mL. The cytokine was replenished twice weekly over a period of 3-4 weeks, during which time non-adherent blastic cells were observed to vigorously proliferate in suspension. After 1.5-2 weeks of culture, gradual emergence of adherent single cells and ostensibly clonal foci was observed. After 2.5 weeks, suspension cells were split 1:3, leaving adherent cells on the plastic surface in fresh medium. Continued proliferation was observed among non-adherent wells and to a lesser extent among adherent cells; by 3-4 weeks of culture, adherent wells depleted of suspension cells appeared static and were trypsin harvested for cryopreservation. Progressive differentiation was observed in wells with non-adherent cells, which gradually adhered to the plastic over an additional 2-3 passages. By six weeks, all cells were trypsin harvested and cryopreserved.

Lymphoid cells including Molt4-, SupT1-, CEM-, and 8E5-derived cell lines were propagated in RPMI containing 2X L-glutamine, 1X sodium pyruvate, 1X HEPES, 1X Pen/Strep, and 10% fetal bovine serum. Cultures were maintained between 105

and 2x206 cells/mL, and split 1:10 or 1:15 twice weekly. For aggressive subcultures described using 8E5 cells, confluent cultures were split 1:50 and allowed to recover over 1.5-2 weeks, when the process was repeated. For co-culture experiments using lymphoid cell lines and adherent cells, RPMI was used in place of DMEM medium to accommodate lymphocyte culture preferences. Molt4R5-derived cells were maintained under 1mg/mL Geneticin selection. SupT1/R5-derived cells were maintained under 3µg/mL blasticidin selection.

Transformed adherent cell lines were maintained in DMEM complete medium 2X L-glutamine, 1X sodium pyruvate, 1X HEPES, 1X Pen/Strep, and 10% fetal bovine serum. Affinofile-GGR cells were maintained under 50µg/mL blasticidin selection, while 293FT cells were periodically selected using 500µg/mL Geneticin.

Viruses

HIV clones JR-CSF, JR-FL, and pWT-BaL, and a panel of Clade B transmitted/founder viruses were acquired through the NIH AIDS Reagent Program. Clade C T/F clones were graciously offered by Dr. John Kappes (UAB). HIV clone ADA and stock of BaL were graciously provided by Mario Stevenson, Miami CFAR. Tagged HIV-nef-IRES-GFP (BaL env) was a gift of Thorsten Mempel, MGH, and Thomas Murooka, U.Manitoba. Tagged HIV-gag-imCherry (BaL) was generously offered by Phillipe Benaroch, institutCurie, and Mike Schindler, Helmholtz Center Munich. HIV-nef-IRES-mCherry was generated by cloning the mCherry cDNA into HIV-nef-IRES-GFP, using PCR amplification from pCAAGS-mCherry (a kind gift of

Natasza Kurpios) and cloning a 1.7kb MluI-IRES-mCherry-LTR-XbaI cassette into digested pNL4-3-nef-IRES-GFP(BaL).

Production of virus stocks

Infectious molecular clones of HIV were transfected into 293FT cells without helper plasmids (for wild type HIV) or with pLP-VSV/G (Invitrogen) to produce pseudotyped virions. Transfections of 80-90% confluence 293FT were carried out on day one in T-150 culture flasks using Lipofectamine 2000 according to the manufacturer instructions, using 40µg of HIV DNA ± 5 µg of VSV/G DNA. After overnight incubation, medium was replaced on day two with fresh DMEM complete medium containing Pen/Strep and cultured for 24 additional hours. Harvest of supernatant was made on days three and four, with day three supernatant stored at 4°C overnight. On day four, supernatants were pooled, cleared by centrifugation at 1000 RCF for 10 minutes at 4°C, filtered through a 0.45uµM PVDF ultra low protein-binding filter (Millipore) and aliquoted for storage at -80°C. Viral titer was established on single freeze-thaw cycle stocks.

Lentiviral vector construct pNL-GFP-RRE(SA) was packaged in a similar fashion, with the addition of plasmid DNA pCMV-dR8.2-dvpr to express Rev, Gag/pol, and Tat. To package pNL-GGR-RRE(SA), Affinofile-GGR cells were used as packaging cells given that the GGR plasmid clone was unavailable. Affinofile-GGR cells were transfected with pCMV-dR8.2-dvpr and pLP-VSV/G to package the GGR provirus integrated in these cells. To overcome the low lentiviral output (single copy

proviral transcription vs. plasmid DNA overexpression), target cells were mixed with Affinofile-GGR cells budding GGR lentivirus and spinoculated at 1000xg for 2 hours at room temperature. Target cells were then separated from packaging Affinofile-GGR cells by limiting dilution and cloning. Low-titer GGR lentiviral stock was also archived from the Affinofile-GGR supernatants.

Infections

Inoculations were performed as described above, with modifications. HMDM were infected in adherent culture or in Teflon screwtop jars (Savillex), without added DEAE-dextran or polybrene. Inoculum medium was changed after 24-48 hours, and cells incubated for 4-14 days for viral replication. HMDM cultures were never infected before day 7 of differentiation.

Adherent reporter cells were infected with varying doses wild type or VSV-pseudotyped viral stocks without DEAE dextran. Medium was changed after 24-48 hours and cells followed for reporter gene expression.

Non-adherent lymphoid cell lines were infected with HIV or lentiviral stocks using spinoculation in 20 µg/mL DEAE-Dextran, except where indicated above. For experiments directly comparing infection in adherent or HMDM cultures, lymphoid reporter cells were infected in standing culture without spinoculation or polycationic culture additives.

Absolute titer assay

Stocks of wild type infectious molecular clones were tittered on a panel of cell lines. To 2,500 target cells in 96-well format was added 50µL of tenfold serially-diluted viral stocks. Target cells were 10 day-old HMDM or SupT1/CCR5 cells, cultured without DEAE-dextran. Target wells with medium only (no target cells) were plated simultaneously to test viral decay in the period prior to reporter cell addition. Target cells were cultured for 5 days without medium changes to allow viral infection. On day 5, 10,000 SupGGR cells were added to each well to quantify the presence of HIV in target wells compared to medium only wells. For each viral dilution, the percentage of positive wells was established by reporter assay, and the fraction of positive wells used to discern the TCID50 for each viral stock. Background correction was performed for each viral dilution by subtracting the TCID50 dose in medium-only wells from that in target wells.

Single cell VOA

Bulk cultures of donor cells were infected with relevant strains of infectious HIV. During proof of concept experiments, 5 x 10^4 TZM-gfp cells were infected with lab adapted strain BaL or primary Clade C swarm 27ZP for 5 days. Cells were trypsin harvested on day 5, strained through a 40 µm nylon cell strainer (Fisher) to remove large syncytia and aggregates, and an aliquot quantified for GFP penetrance on a BioRad S3e cell sorting instrument. The original (unsorted) bulk cells were diluted to 0.75 cells per 50µL of fresh DMEM complete medium, and 50µL of cell suspension

were dispensed into each well containing 10,000 pre-seeded MoltGGR cells using a multichannel pipette. Cultures were incubated for 8 days, with longitudinal sampling performed on days 3 and 8 by removing 20µL of medium from the top of each well with a multichannel pipette. GLuc assay was performed according to the manufacturer's instructions (Thermo), using an integration time of 0.1s in an Ultra sensitivity luminescence protocol on an Envision multilabel plate reader (Perkin Elmer). Data were plotted in Prism 6.0 on log box and whisker plots, using Tukey 1.5x IQR criteria to score positive outliers.

For single cell viral outgrowth assays using HMDM, HuFLM, and SupT1/R5, these target cells were infected with VSV-HIV-IRES-mCherry stocks for 3-5 days in bulk culture on untreated 6-well dishes (Corning). The afternoon prior to harvest, optical bottom 96-well plates of outgrowth reporter cells were seeded with 5,000 Affinofile-GGR, TZM-GGR, or TZM-bl cells; the Affinofile GGR cells were seeded in maximum induction medium containing 4.0 ng/mL of Doxycycline and 2 µM of Ponasterone A. On the day of sorting and co-culture, outgrowth cells were replenished with fresh medium. Adherent target cells (HMDM, HuFLM) were trypsin harvested as described above, while SupT1/R5 cells were collected by simple pipetting. Harvested cells were filtered using 40-70µm nylon cell strainers (Fisher) prior to purification of the mCherry-positive fraction on a BioRad S3e FACS instrument. Purified cells were pelleted and resuspended in fresh complete medium at a theoretical concentration of 5 cells per 50uL. To each well of the reporter plates was added 50µL of target cell suspension. Cultures were incubated for 5 days, and

followed for reporter gene expression by confocal microscopy starting 48 hours after co-culture.

Drop culture

TZM-gfp cells were trypsin harvested and resuspended at ~107/mL in a sterile microfuge tube. A series of 20 twofold dilutions was prepared, with dilutions down to 20 cells/mL (0.04 cells per 2µL). From each dilution tube and starting from the most dilute, 2µL were spotted onto the surface of a 35mm glass-bottom plate (ibidi) in a 5 x 5 spot array. The plate was sealed and incubated overnight in a cell culture incubator to allow adherence of foci in each drop. The following morning, the plate was washed with fresh medium and inoculated with wild type HIV-1 ADA supernatant, such that all foci shared the same viral supernatant. Cultures were incubated for 72 hours, and each cell focus analyzed for HIV infection by confocal microscopy.

Primary HIV capture – ***performed by collaborators in Blantyre, Malawi***

Twenty-four hours prior to BAL harvest, 200,000 TZM-gfp or Molt4R5 cells were plated in 6-well format by team members in Blantyre. On the day of BAL harvest, 250,000 bulk lavage cells were added to TZM-gfp or Molt4R5 cultures, including TZM cultures on glass coverslips for electron microscopy. At 24 or 48 hours post-inoculation, culture supernatant was harvested and cleared by centrifugation, while adherent cells were trypsinized and viably archived in freeze medium containing 10% DMSO and 90% FBS. Non-adherent Molt4R5 cells were pelleted and

similarly archived. Supernatants and cells were stored at -80°C and shipped to Ithaca, NY on dry ice. Coverslips were submerged in fixative containing 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.2. in a Wash-N-Dry coverslip rack (Diversified Biotech), and sealed in a teflon jar (Savillex) for shipping to Ithaca, NY at room temperature.

Secondary outgrowth – *performed in the Russell Lab, Ithaca, NY*

Secondary cell co-cultures were initiated by thawing viably archived primary co-culture cells, diluting 1:10 in fresh medium and pelleting cells at 200x g for 5 minutes. Pellets were resuspended in fresh DMEM complete medium, and were (1) placed into co-culture with maximally-induced Affinofile-GGR cells seeded the afternoon prior, (2) spinoculated with MoltGGR cells in the presence of 20µg/mL DEAE dextran, or placed into viable monoculture. A-GGR and MoltGGR cells were followed by fluorescence microscopy over 10 days. No reporter signal of any kind emerged in the three culture formats, including the primary TZM-gfp monoculture cells. Cells and supernatants were harvested for RNA PCR analyses as described below.

Secondary supernatant outgrowth cultures were initiated by thawing aliquots of primary viral supernatants, and inoculating maximally-induced Affinofile-GGR cells or spinoculating Molt4R5, or MoltGGR cells in the presence of 20µg/mL DEAE-dextran. Outgrowth cultures were followed by fluorescence microscopy over a 13-day period where lymphoid outgrowth cultures were harvested and

centrifuged (200xg) every other day for the first week. On day 5, A-GGR co-cultures were re-induced with Doxycycline and Ponasterone A as described above, incubated 2 additional days to allow a single round of viral replication, then supernatants and floating cells were passaged onto freshly-seeded and max–induced A-GGR cells for an additional 7 days of co-culture. On day 13, supernatants were harvested and cells lysed in TRIZOL for PCR amplification of viral RNA. On day 13, overt and logarithmic infection was observed in 10.27Z-PBMC outgrowth cultures. Fresh Molt4R5 cells were spinoculated with the Molt4R5 culture for this 10.27Z-PBMC sample, and the culture was extended for a third week. Infectious viral supernatant was harvested on day 21, 0.45µm PVDF-filtered, and aliquoted for storage at -80°C as "27ZP stock".

Electron Microscopy – *performed in the Russell Lab by Shannon Caldwell*

Upon their return to Ithaca, coverslips were rinsed 3x10 minutes in sodium cacodylate buffer, then fixed for 1hour in buffered 2% osmium tetroxide. Three x 5 minute rinses in sodium cacodylate buffer were followed by a single 5-minute rinse in distilled water and 20-minute soak in 1% aqueous uranyl acetate. Coverslips were rinsed again in distilled water, then dehydrated in a 10-minute graded aqueous ethanol series of 10, 30, 50, 70, 90, 100, and 100% ethanol. A graded ethanol: Ultra bed resin infiltration series was performed: 4:1, 20 minutes; 2:1, 60 minutes; 1:1, overnight; 1:2, 3 hours; 1:4, 4 hours; 0:1, overnight (air exposed); and finally 0:1, 4 hours. Infiltrated coverslips were then inverted over the end of a Beam capsule filled with resin and polymerized overnight at 65-70°C. Embedded specimens were

processed through hydrofluoric acid to dissolve glass coverslips. Blocks were cleaned and trimmed, ultrathin sections were cut and placed on formvar coated grids. Grids were stained for 20 minutes in uranyl acetate, 7 minutes in lead citrate, then dried and imaged on an FEI Tecnai 12 Bio-twin transmission electron microscope.

FISH:FLOW

Live cells were harvested by BAL, venipuncture, or from cell culture. Single cell suspensions were generated, rinsed in PBS, and fixed in 1% PFA for 30 minutes. Cells were pelleted at 2500 RPM in a swinging bucket Hermle Z200 microfuge, and resuspended in 70% ethanol in RNAse-free water overnight for permeabilization or long-term storage. For FISH staining, cells were pelleted, the ethanol was discarded, and 1mL of FISH wash buffer was added: 2X SSC (Ambion), 1X Denhardt's solution (Affymetrix), 10% deionized formamide (EMD Millipore) in nuclease-free water. After standing for 5 minutes in wash buffer, cells were pelleted and resuspended in 100µL hybridization buffer with 1mg/mL E.coli tRNA or a modified buffer lacking tRNA but containing 1mg/mL unlabeled, scrambled ssDNA oligonucleotide, "X-buffer". H- and X-buffer were otherwise identical, containing 200 mg/mL of dextran sulfate (Amresco), 2mM vanadyl ribonucleoside complex (NEB), 200µg/mL RNAse-free BSA, 2X SSC, 10% deionized formamide in nuclease-free water. To 100µL hybridization reactions was added 1µL of FISH probe (LGC Biosearch technologies, final concentration 2.5-250 nM). Tubes were sealed and incubated at 37°C in a tissue culture incubator in the dark overnight. The following day, 1mL of FISH wash buffer was added, left to stand for 5 minutes in the dark, then centrifuged at 2000 RPM.

Wash solution was discarded, and replaced with another 1mL wash buffer. Tubes were sealed and incubated at 37°C for 30 minutes, then pelleted and resuspended in PBS containing 1% BSA for flow cytometry. Biosearch probes targeting HIV-1 gag, nef, 3'LTR, and 5'LTR or SIVmac239 nef and 5'LTR were ordered with various flurophores including Quasar570, Quasar670, and CalFluor Red 590.

Confocal imaging

Image analyses of BSL-3 HIV infections were performed on a Leica SP-5 laser scanning confocal microscope using either a universal plate holder for glass-bottom or chamber glass culture formats, or the H201–MEC–LG–MW holder (OkoLabs) for optical bottom 96-well plate culture assays. Z-stacks were projected, and channels merged contrasted in Leica Application Suite or Adobe Photoshop.

P24 ELISA

Supernatant samples were applied to the HIV Type 1 p24 Antigen ELISA (Zeptometrix) according to manufacturer instructions. Infectious HIV was neutralized using the included lysis buffer, and the ELISA was performed using a dilution series of known standards of purified p24 protein.

HIV env and gag PCR – *Performed in the Russell Laboratory by Saikat Boliar with contributions from David Gludish*

HIV sequences were amplified using nested PCR approaches. Cellular mRNA or viral RNA in supernatant was isolated using TRIZOL or the Qiamp viral RNA mini kit,

respectively per manufacturer's instructions. Reverse transcription was performed using the SuperScriptIII kit (Invitrogen) and the ofm19 RT primer 5'- GCA CTC AAG GCA AGC TTT ATT GAG GCT TA. Envelope PCR was performed using Phusion Hi-Fidelity polymerase in two steps: (1) ofm19 rev with Vif1 fwd, for 40 cycles at 55°C annealing, and (2) EnvA with EnvN, for 40 cycles at 55°C annealing. A portion of the first step product was used as template in the second step reaction. From the same ofm19 cDNA libraries, gag PCR was performed in two steps: (1) OuterFor with OuterRev for 40 cycles at 55°C annealing, and (2) InnerFor with InnerRev for 40 cycles at 55°C annealing, again using a portion of the first step product as template in the second step.

PCR products were gel-purified, cloned using the Zero blunt TOPO PCR cloning kit (Invitrogen), and sequenced.

PCR primers:

ofm19 rev 5'- GCA CTC AAG GCA AGC TTT ATT GAG GCT T

Vif1 fwd 5'- GGG TTT ATT ACA GGG ACA GCA GAG

EnvA 5'- CAC CGC CTT AGG CAT CTC CTA TGG CAG GAA GAA

EnvN 5'- CTG TCA ATC AGG GAA GTA GCC TTG TGT

OuterFor 5'- AAGTAAGACCAGAGGAGATCTCTCGAC

OuterRev 5'- GACAGGTGTAGGTCCTACTAATACTGTACC

InnerFor 5'- TTTGACTAGCGGAGGCTAGAAGGA

InnerRev 5' - GTATCATCTGCTCCTGTGTCTAAGAGAGC

RNAseq analyses

FISH-sorted cells were pelleted and resuspended in PKD buffer from the Qiagen RNeasy FFPE kit. The remaining steps from the Microdissected FFPE tissue sections protocol were followed. Approximately 100 ng RNA was used to prepare a cDNA library using the Ovation Human FFPE RNA-Seq Library System, according to the manufacturer's protocol. To account to degradation suffered by PFA fixation, RNA is reverse transcribed using a mixture of random and poly(T) primers. The completed preparation yielded an rRNA depleted, strand-specific, adaptor ligated cDNA library suitable for Illumina NGS. Sequencing was performed in single-end 50 bp lanes on a Illumina HiSeq 2000/2500 (high-output mode). RNA sequencing reads were aligned to the HIV reference genome using the TopHat algorithm within Geneious software and analyzed for coverage.

APPENDIX 1: Cell lines used in this study

	CELL LINE	ABBREVIATION	DESCRIPTION
Macrophage	THP-1	**THP-1**	Monocyte-macrophage cell line
	HMDM	**HMDM**	Primary human monocyte-derived macrophages
	HuFLM	**HuFLM**	Primary human fetal liver-derived macrophages
HeLa	HeLa	**HeLa**	Human cervical cancer cells
	JC-53	**JC-53**	HeLa derivative Stable expression of: CD4, CCR5 and CXCR4
	JC53-bl	**TZM-bl**	JC-53 HeLa derivative HIV-LTR responsive **b-gal and luciferase reporter**
	TZM-gfp	**TZM-gfp**	JC-53 HeLa derivative HIV Tat/Rev-responsive **GFP reporter**
	TZM-GGR	**TZM-GGR**	JC-53 HeLa derivative HIV Tat/Rev-responsive **GFP and secreted gaussia luciferase reporter**
HEK293	HEK293	**293**	Human embryonic kidney cells
	293FT	**293FT**	HEK293-derivativeWith SV40 largeT antigen
	Affinofile-GGR	**A-GGR**	Drug-inducible CD4 and CCR5 HIV Tat/Rev- responsive **GFP and secreted gaussia luciferase reporter**
Molt4	Molt4	**Molt4**	Human T lymphoblast [from acute lymphoblastic leukemia cells]
	Molt4/CCR5	**M4R5**	CCR5-transfected Molt4 T cells
	Molt4/R5-GGR	**MoltGGR**	Molt4 cells with HIV Tat/Rev- responsive **GFP and secreted gaussia luciferase reporter**
SupT1	SupT1	**SupT1**	T lymphoblastic lymphoma cell line
	SupT1/CCR5	**SupR5**	CCR5-transfected SupT1 cells
	SupT1/R5-GFP	**SupGFP**	SupT1/R5 cells with HIV Tat/Rev-responsive **GFP reporter**
	SupT1/R5-GGR	**SupGGR**	SupT1/R5 with H IV Tat/Rev- responsive **GFP and secreted gaussia luciferase reporter**
CEM	CEM	**CEM**	Acute lymphoblastic leukemia CD4 / CXCR4 expression
	CEM-GFP	**CEM-GFP**	CEM-derivative HIV LTR-responsive GFP reporter
	8E5/LAV	**8E5**	CEM-derivative HIV-infected with POL lesion Single HIV copy per cell

reporter cell line

constructed in this study

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